Rejections under 35 U.S.C. §§ 101 and 112, First Paragraph

The Examiner maintains rejection of claims 25-79 under 35 U.S.C. § 101 as allegedly not being "supported by either a specific and substantial asserted utility or a well-established utility." In particular, the Examiner alleges that Applicants assertions are insufficient "because Applicants have failed to demonstrate the effect of the claimed protein on cells." *See*, Paper No. 20, pages 2-3. Applicants respectfully disagree and traverse this rejection.

A rejection under 35 U.S.C. § 101 is improper when a person of ordinary skill in the art would find credible disclosed features or characteristics of the invention, or statements made by the applicant in the written description of the invention. *See*, M.P.E.P. §§ 2107.02(II), (III) at 2100-[37-39] (Original Eighth Edition, Aug. 2001). In addition, an applicant need only make *one* credible assertion of utility for the claimed invention to satisfy 35 U.S.C. § 101. *See, e.g., Raytheon v. Roper,* 724 F.2d 951, 958, 220 U.S.P.Q. 592, 598 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 835 (1984) ("When a properly claimed invention meets at least one stated objective, utility under 35 U.S.C. § 101 is clearly shown."). *See*, M.P.E.P. at 2100-37. Finding a lack of utility is also improper if a person of ordinary skill in the art would know of a use for the claimed invention at the time the application was filed. *See*, M.P.E.P. § 2107.02(II)(B) at 2100-[38-39].

Moreover, the burden is on the Examiner to establish why it is more likely than not that one of ordinary skill in the art would doubt *(i.e.,* "question") the truth of the statement of utility. *See*, M.P.E.P. § 2107.01(III)(A) at 2100-[39-40]. Thus, the Examiner must provide evidence sufficient to show that the statement of asserted utility would be considered "false" by a person of ordinary skill in the art. *Id.* The Examiner must also present countervailing facts and reasoning sufficient to establish that a person of ordinary skill would not believe the applicants' assertion of utility. *See id.*; *see also, In re Brana,*

51 F.3d 1560, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). For the reasons set forth below, the Examiner has not met the burden that is necessary to establish and maintain a rejection for lack of utility under 35 U.S.C. § 101.

However, contrary to the Examiner's comments, Applicants have set forth in the specification statements that clearly and fully describe the function of Human Cytokine Polypeptide of the present invention and explain why Applicants believe the invention is useful. For example, the specification, at page 3, lines 5-8, teaches that polypeptides of the present invention may be used to treat inflammation. Applicants contend that specific and substantial utilities have been disclosed in the specification as filed and that the only issue is whether any asserted utility is credible.

Inflammation is a result of the inflammatory response, which occurs when a tissue is exposed to any one of a number of noxious stimuli including, for example, bacterial infections. (*See*, e.g., Exhibit A, Janeway, C.A. and Travers, P. 'IMMUNOBIOLOGY – The Immune System in Health and Disease', Garland Publishing Inc., N.Y. and London, at pages 1:30 to 31 (1994)). As described by the authors, the initial inflammatory response is characterized by certain specific events including blood vessel dilation, local increase in vessel stickiness for passing immune cells, and increased vessel permeability to fluid and immune cells. The first cells involved in the inflammatory response are non-specific 'inflammatory cells' such as monocytes and neutrophils; however, later recruitment and activation of T cells may serve to sustain chronic inflammation.

T cells are recognized as playing a significant role in the maintenance of the inflammatory response, for example, through the secretion of pro-inflammatory cytokines. (*See*, e.g., Exhibit B, Abbas, A.K., Lichtman, A.H. and Pober, J.S. 'Cellular and Molecular Immunology', W.B. Saunders Company, Philadelphia, at pages 239 to 240 (1991)). Abbas et al. describe "cytokines that activate inflammatory cells" including

gamma interferon, lymphotoxin, interleukin-5 and migratory inhibition factor as all being produced by T cells. These cytokines serve to mobilize and/or activate numerous cells involved in the inflammatory response including, for example, mononuclear phagocytes, endothelial cells, neutrophils, eosinophils and B cells. (*See,* Table 11-3, *Id.* at page 239).

Maturation and selection of T cells occurs in the thymus and continues into adult life. (*See*, e.g., Exhibit C, Abbas, A.K., Lichtman, A.H. and Pober, J.S. 'Cellular and Molecular Immunology', W.B. Saunders Company, Philadelphia, at pages 23 to 26 (1991)). The authors note that no "extrathymic sites of T cell development have been identified." (*See*, e.g., *Id.* at page 26, left column at lines 9-10).

Accordingly, in light of what was widely known and accepted in the art, at the time of filing of the instant application one of ordinary skill in the art would have accepted that: (1) inflammation is a key mechanism used to isolate, restrict and combat the effects of harmful agents; (2) T cells play a coordinating role in the initiation and maintenance of the inflammatory response; and (3) T cells mature and are selected only in the thymus from birth through adulthood.

In support of the asserted utility of the present invention in the treatment of inflammation, Applicants respectfully direct the Examiner's attention to the teachings of Graf et al. (*J. Exp. Med.*, 196(2): pp 163-171. (2002)) who show that polypeptides of the present invention are expressed and play a role in the regulated development of T cells in the thymus. A legible copy of Graf et al. is enclosed herewith as Exhibit D.

Graf et al. demonstrate that Tsg expression is upregulated after T-cell receptor activation in developing thymocytes (*See*, Figure 1), that the components of the BMP signaling pathway are expressed in the thymus (*See*, Figure 2), and that BMP4 directly inhibits thymocyte development and proliferation (*See*, Figure 3). The authors then demonstrate that BMP4 inhibition of thymocyte development cannot be prevented by

either Tsg or chordin alone, but that together Tsg and chordin are able to completely reverse this inhibition (*See*, Figure 5). Graf et al. propose that the coordinated expression of Tsg and chordin in the thymus is required for proper T-cell development and "the balance between BMP2/4, chordin, and Tsg may ensure developmental progression while maintaining a sufficient pool of immature precursors." *See*, page 170, left column, lines 1-3. Therefore, given the central role of T-cells in immune system function and inflammation, and in view of the teachings of the specification as filed, supported by those of Graf et al. (Exhibit A), Applicants maintain that the present invention is useful as required under 35 U.S.C. § 101.

The Examiner maintains rejection of the pending claims under 35 U.S.C. § 101, as allegedly "this member of the protein family does not have a specific utility because the specific function of this particular protein has not been demonstrated." *See*, Paper No. 20, page 4.

Knowledge of a biological or pharmacological activity of a compound is beneficial to the public, and "adequate proof of any such activity constitutes a showing of practical utility." *Nelson v. Bowler*, 626 F.2d 853, 856 (C.C.P.A. 1980). Applicants disclose in the specification the credible assertion that the Human Cytokine Polypeptide of the present invention may be useful to treat inflammation. Moreover, Applicants have provided evidence that the Human Cytokine Polypeptide of SEQ ID NO:2 regulates T-cell development, a critical aspect of inflammation. As such, Applicants submit that adequate proof of a biological activity of the Human Cytokine Polypeptide has been shown, thereby constituting a showing of practical utility.

In view of the above, the assertions that Human Cytokine Polypeptide would act to regulate immune cell development and homeostasis, and would be useful as a means of treatment in inflammation, would *not* be incredible to one skilled in the art. That is, since

the facts upon which the assertions are based are consistent with the logic underlying the assertions, Applicants submit that one of ordinary skill in the art would *not* reasonably doubt Applicants' assertions regarding utility.

Utility can exist for therapeutic inventions "despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or bioactive compound or composition." M.P.E.P. § 2107 (III) at 2100-27. "Usefulness in patent law . . . necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." *In re Brana*, 51 F.3d 1560, 1568 (Fed. Cir. 1995).

Further, Applicants do not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty or provide actual evidence of success in treating humans where such a utility is asserted. *See* M.P.E.P. § 2107.02 (I) at 2100-34. All that is required of Applicants is that there be a *reasonable* correlation between the biological activity and the asserted utility. *See Nelson v. Bowler*, 626 F.2d at 857.

Based upon the general knowledge of those skilled in the art that the regulation of immune cell homeostasis is directly implicated in the development of inflammation, it would be reasonable to expect that Human Cytokine Polypeptide of the present invention could be utilized in the treatment of inflammation. As such, based on the totality of the evidence, an artisan of ordinary skill in the art of molecular biology would find the statements of utility contained in the specification to be credible.

Applicants further point out that the patentability of the present invention, based on this utility, is <u>not</u> dependent on disclosure of the details of how or why the invention

works. The Federal Circuit has recently stated with respect to the rejection of claims for lack of utility that:

> "It is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works." Newman v. Quigg, 877 F.2d 1575, 1581, 11 U.S.P.Q.2D (BNA) 1340, 1345 (Fed. Cir. 1989); see also Fromson v. Advance Offset Plate, Inc., 720 F.2d 1565, 1570, 219 U.S.P.Q. (BNA) 1137, 1140 (Fed. Cir. 1983) ("It is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests."). Furthermore, statements that a physiological phenomenon was observed are not inherently suspect simply because the underlying basis for the observation cannot be predicted or explained.

In re Cortright, 49 U.S.P.Q.2d 1464, 1466 (Fed. Cir. 1999). Likewise, according to the axiom of patent law, the utilities asserted for the Human Cytokine Polypeptide do not depend on identification of any receptor or cofactor necessary for its biological activity. Rather, the issue is whether an asserted utility is true.

Even assuming, *arguendo*, the Examiner has established a *prima facie* showing that the claimed invention lacks utility, Applicants respectfully submit that they have rebutted the Examiner's showing by proffering sufficient evidence to lead one skilled in the art to conclude that the asserted utilities are more likely than not true. Applicants have supplied evidence that the Human Cytokine Polypeptide of SEQ ID NO:2 is identical to a molecule, *i.e.*, Tsg, that acts as a regulator of T-cell development and homeostasis and is likely involved in inflammation.

In view of the facts set out above, Applicants assert that a skilled artisan would not reasonably doubt that polypeptides comprising the Human Cytokine Polypeptide amino acid sequence shown in SEQ ID NO:2 can be used in the treatment of inflammation. As such, Applicants assert that the presently claimed invention possesses a credible utility that constitutes a patentable utility under 35 U.S.C. § 101.

In view of the above, Applicants submit that the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101. The Examiner has failed to establish and maintain grounds as to why a rejection for lack of utility is proper. Accordingly, Applicants respectfully request that the rejection be withdrawn.

The Examiner has also rejected claims 25-79 under 35 U.S.C. § 112, first paragraph, "since the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention".

Applicants respectfully disagree and traverse this rejection.

As detailed above, the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101 and, armed with the specification of the instant invention, one skilled in the art clearly would know how to use the claimed invention. Accordingly, Applicants respectfully request that the rejection of claims 25-79 under 35 U.S.C. § 112, first paragraph, be withdrawn.

Conclusion

Applicants respectfully request consideration and entry of the foregoing remarks into the file. Applicants believe that no fee is due in connection herewith; however, should the Patent Office determine otherwise, please charge the required fee to Human Genome Sciences, Inc., Deposit Account No. 08-3425.

Respectfully submitted,

Dated: May 9, 2003

Janet M. Martineau (Reg. No. 46,903)
Attorney for Applicants

Human Genome Sciences, Inc.
9410 Key West Avenue
Rockville, MD 20850
(301) 315-2723 (telephone)

Enclosures
JMM/BM

IMMUNOBIOLOGY

THE IMMUNE SYSTEM IN HEALTH AND DISEASE

Charles A. Janeway, Jr.
Yale University Medical School

Paul Travers
Birkbeck College, London University

CB
CURRENT
BIOLOGY
LIMITED

Current Biology Ltd
London, San Francisco and Philadelphia

Blackwell
Scientific
Publications
OXFORD

Garland Publishing Inc
New York and London

List of headings

Part I AN INTRODUCTION TO IMMUNOBIOLOGY

Chapter 1: Basic Concepts in Immunology

Chapter 2: The Induction, Measurement, and Manipulation of the Immune Response

Many microorganisms, especially bacteria, have conserved surface molecules that are recognized by phagocytic cells, which play an important part in the early elimination of infection as well as serving as professional antigen-presenting cells and thereby inducing the later adaptive immune responses. These phagocytic cells include macrophages and neutrophils, which not only ingest and destroy extracellular microorganisms, and in particular bacteria, but are also important in recruiting other cells and molecules of the immune system by releasing chemicals that have effects collectively called inflammation.

1-19 Infection often triggers an inflammatory response.

The term **inflammation** is purely descriptive and was originally defined by the four Latin words *dolor*, *rubor*, *calor*, and *tumor*, meaning pain, redness, heat, and swelling. These changes result from changes in the local blood vessels, leading to their dilation, increased permeability, and increased stickiness for passing leukocytes and lymphocytes. The increased blood flow accounts for the heat and redness, while the leakage of cells and fluids into the tissue and their local actions account for the pain and swelling. The main cell types seen in inflammatory responses are polymorphonuclear neutrophilic leukocytes together with macrophages and their precursor monocytes; these are therefore known as **inflammatory cells**. Lymphocytes, as well as small numbers of eosinophils and basophils, also accumulate at sites of inflammation and, when extreme vascular leakage occurs, red blood cells may also occasionally be found. Inflammatory responses can be triggered directly by pathogens, especially bacteria, early in infection, and may be sustained later by antibodies and by T cells, which release inflammatory factors. In the early phase of an infection, inflammatory responses are important in attracting nonspecific inflammatory cells such as monocytes and neutrophils to the site of an infection. Later, the same changes attract effector lymphocytes, and

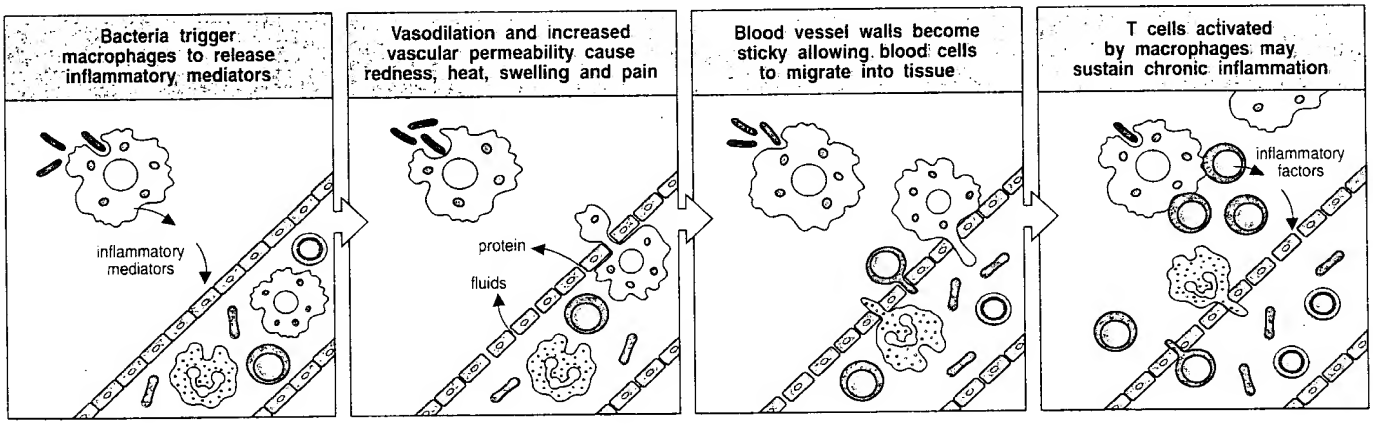

Fig. 1.32 Bacterial infection triggers an inflammatory response. Macrophages encountering bacteria in the tissues are triggered to release chemicals that increase the permeability of blood vessels, allowing fluid and proteins to pass into the tissues. The stickiness of the endothelial cells of the blood vessels is also changed, so that cells adhere to the blood vessel wall and are able to crawl through it: macrophages and polymorphonuclear neutrophilic leukocytes (neutrophils) are shown here entering the tissue from a blood vessel. The accumulation of fluid and cells at the site of infection causes the swelling, heat, and pain that are collectively known as inflammation. Macrophages and neutrophils are the principal inflammatory cells. Later in an immune response, activated lymphocytes also contribute to inflammation.

the increased permeability of the blood vessels allows the passage of antibodies into infected tissues (Fig. 1.32).

Many different stimuli can trigger inflammatory responses. Physical injury from wounds or burns releases proteins from tissues that trigger acute inflammatory reactions similar to those activated directly by bacteria. Chronic inflammatory processes are usually triggered by T cells, especially those that activate macrophages, as activated macrophages frequently cause local tissue damage through the release of mediators similar to those elicited by bacteria. Finally, some forms of acute inflammatory response are triggered by specific antibodies binding to antigen and activating the complement system, or interacting with accessory cells through their receptors for bound antibody molecules, as we shall see in the next section. The inflammatory response is a general term to describe both the gross and microscopic picture of local tissue infiltration by fluid and cells triggered in these different ways.

1-20 Specific recognition of pathogens by antibodies activates non-specific accessory cells.

Many microorganisms have evolved adaptations to their surface molecules that enable them to escape direct detection by any of the innate mechanisms we have described above. These microorganisms must be recognized by lymphocytes whose diverse receptors enable them to detect any pathogen and mount an adaptive immune response. The mechanisms whereby microorganisms are then destroyed, however, are essentially the same for the innate and adaptive arms of the immune response.

Thus, bacteria that resist direct binding by complement and are not bound by acute-phase proteins can become coated with specific antibodies. Once the antibodies have bound to the bacterium, they in turn recruit complement (see Fig. 1.22), as well as **accessory effector cells** that have receptors for bound antibody and complement molecules. These effector cells are the same as those that participate in innate immunity, and thus antibody, by flagging a pathogen as foreign, is able to overcome the ability of some pathogens to evade innate immune mechanisms. The accessory cells and the mechanisms whereby they eliminate pathogens are summarized in Fig. 1.33; we shall learn more about these cells when we discuss humoral immunity in Chapter 8.

Similarly, T cells recognize antigen specifically, but then trigger effector mechanisms that are not antigen specific. Specificity in cell-mediated immunity comes from the antigen-specific release of antigen-nonspecific effector molecules. Thus, killer CD8 T cells release their cytotoxic molecules only when they encounter an infected host cell, and inflammatory CD4 T cells activate only infected macrophages. Only in the case of B-cell activation by helper T cells is the target of T-cell action also antigen specific; however in this case, as we have just seen, the effector mechanism ultimately activated by the antibodies will not be antigen-specific. Thus, in both humoral and cell-mediated immunity, specificity derives from the clonally distributed receptors on antigen-specific lymphocytes, while effector function is mediated by cells and molecules that are not specific for antigen. This allows the same effector mechanisms to be used in response to a wide range of distinct pathogens.

Principal text editor: Miranda Robertson
Text editors: Rebecca Ward, Eleanor Lawrence
Project editor: Rebecca Palmer
Assistant project editor: Emma Dorey
Principal designer and illustrator: Celia Welcomme
Designer: Sylvia Purnell
Assistant illustrator: Matthew McClements
Production: Rebecca Spencer
Graphics software support: Gary Brown
Proofreader: Melanie Paton
Indexer: Nina Boyd
Photo research: Doug McGaughy, Tamsin Newmark

Distributors
Inside North America: Garland Publishing Inc., 717 Fifth Avenue, New York, NY 10022, USA.
Inside Japan: Nankodo Co. Ltd., 42-6, Hongo 3-Chome, Bunkyo-ku, Tokyo 113, Japan.
Outside North America and Japan: Blackwell Scientific Publications, Osney Mead, Oxford OX2 0EL. Orders to: Marston Book Services Ltd, PO Box 87, Oxford OX2 0DT, UK.
Australia: Blackwell Scientific Publications Pty Ltd., 54 University Street, Carlton, Victoria 3053.

ISBN 0-8153-1497-3 (hardcover) Garland
ISBN 0-8153-1691-7 (paperback) Garland
ISBN 0-86542-811-5 (paperback) Blackwell

A catalog record for this book is available from the British Library.

Library of Congress Cataloging-in-Publication Data
Janeway, Charles.
Immunobiology: the immune system in health and disease/ Charles A. Janeway, Jr., Paul Travers.
p. cm.
Includes bibliographical references and index.
ISBN 0-8153-1497-3 (hardcover). ISBN 0-8153-1691-7 (pbk.).
1. Immune System. 2. Immunity. I. Travers, Paul, 1956- . II. Title
[DNLM: 1. Immune System--physiology. 2. Immune System--physiopathology. 3. Immunity--physiology. 4. Immunotherapy. QW 504 1994]
QR181.J37 1994
616. 07'9--dc20
DNLM/DLC
for Library of Congress 94-11058
CIP

This book was produced using Ventura Publisher 4.1 and CorelDraw 3.0.

Printed in Hong Kong by Paramount Printing Co. Ltd.

Published by Current Biology Ltd., Middlesex House, 34-42 Cleveland Street, London W1P 5FB, UK and Garland Publishing Inc., 717 Fifth Avenue, New York, NY 10022, USA.

CELLULAR AND MOLECULAR IMMUNOLOGY

ABUL K. ABBAS, M.B.B.S.
Professor of Pathology
Harvard Medical School and Brigham and Women's Hospital
Boston, Massachusetts

ANDREW H. LICHTMAN, M.D., Ph.D.
Assistant Professor of Pathology
Harvard Medical School and Brigham and Women's Hospital
Boston, Massachusetts

JORDAN S. POBER, M.D., Ph.D.
Professor of Pathology and Immunobiology
Yale University School of Medicine
New Haven, Connecticut

W.B. SAUNDERS COMPANY
Harcourt Brace Jovanovich, Inc.
Philadelphia London Toronto Montreal Sydney Tokyo

W. B. Saunders Company
Harcourt Brace Jovanovich, Inc.
The Curtis Center
Independence Square West
Philadelphia, PA 19106

Library of Congress Cataloging-in-Publication Data

Abbas, Abul K.
Cellular and molecular immunology / Abul K. Abbas, Andrew H. Lichtman, Jordan S. Pober.
p. cm.
ISBN 0-7216-3032-4
1. Cellular immunity. 2. Immunity — Molecular aspects. I. Lichtman, Andrew H. II. Pober, Jordan S. III. Title.
[DNLM: 1. Immunity, Cellular. 2. Lymphocytes — immunology. QW 568 A122c]
QR185.5.A23 1991
616.07'9 — dc20
DNLM/DLC

Editor: Martin J. Wonsiewicz
Designer: Paul M. Fry
Production Manager: Peter Faber
Manuscript Editor: Carol Robins
Illustrator: Risa Clow
Illustration Coordinator: Brett MacNaughton
Indexer: Linda Van Pelt

ISBN 0-7216-3324-2

Cellular and Molecular Immunology

Printed in the United States of America.

Last digit is the print number: 9 8 7 6 5 4

CHAPTER ELEVEN

CYTOKINES

stimulates the growth of others. Often, TGF-β can either inhibit or stimulate growth of the same cell type, depending upon culture conditions such as degree of confluence. TGF-β causes synthesis of extracellular matrix proteins, such as collagens, and of cellular receptors for matrix proteins. (The ability of TGF-β to induce extracellular matrix probably underlies its ability to promote cell growth in soft agar.) *In vivo,* TGF-β causes the growth of new blood vessels, a process called angiogenesis.

As a cytokine, TGF-β is potentially important because it antagonizes many responses of lymphocytes. For example, TGF-β inhibits T cell proliferation to polyclonal mitogens or in mixed leukocyte reactions (see Chapter 16) and inhibits maturation of CTLs. It can also inhibit macrophage activation. TGF-β also acts on non-immune effector cells, such as polymorphonuclear leukocytes and endothelial cells, again largely to counteract the effects of pro-inflammatory cytokines. In this sense, TGF-β is an "anti-cytokine" and may be a signal for shutting off immune responses. Signals that cause T cells to synthesize TGF-β may cause them to behave as suppressor cells (see Chapter 10). *In vivo,* certain tumors may escape an immune response by secreting large quantities of TGF-β.

Although TGF-β is largely a negative regulator of immunity, it may have some positive effects as well. For example, in mice, TGF-β has been shown to switch B cells to the IgA isotype and it may therefore be important in the generation of mucosal immune responses that are mediated by IgA.

Cytokines That Activate Inflammatory Cells

We will now discuss a group of cytokines derived principally from antigen-activated $CD4^+$ and $CD8^+$ T lymphocytes that serve primarily to activate the functions of nonspecific effector cells. Thus, these cytokines mediate the effector phase of cell-mediated immune responses. The molecules described in this section are summarized in Table 11-3.

Immune or Gamma Interferon

Gamma interferon (IFN-γ), also called immune or type II interferon, is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains an identical 18 kD polypeptide encoded by the same gene. IFN-γ is produced both by IL-2 secreting $CD4^+$ helper T cells and by nearly all $CD8^+$ T cells. Transcription is directly initiated as a consequence of antigen activation and is enhanced by IL-2. IFN-γ has been detected in profoundly T cell-deficient animals, and the presumed source in this setting is NK cells; however, NK cells appear to be a minor source of IFN-γ in normal individuals.

As its name implies, IFN-γ shares many activities with type I IFN. Specifically, IFN-γ induces an antiviral state and is antiproliferative. However, IFN-γ binds to a unique cell surface receptor, different from that utilized by type I IFN. The IFN-γ receptor is not related structurally to the other receptor families described earlier. More importantly, IFN-γ has several properties related to immunoregulation that separate it functionally from type I IFN.

1. *IFN-γ is a potent activator of mononuclear phagocytes.* It directly induces synthesis of the enzymes that mediate the respiratory burst, allowing macrophages to kill phagocytosed microbes. Along with second signals, such as LPS and perhaps TNF, it allows macrophages to kill tumor cells. Cytokines that cause such functional changes in mononuclear phagocytes have been called **macrophage-activating factors** (MAFs). *IFN-γ is the principal MAF and provides the means by which T cells activate macrophages.* Other MAFs include GM-CSF and, to a lesser extent, IL-1, TNF, and, in the mouse, IL-4. Macrophage activation is described in more detail in Chapter 12. It is worth noting here that macrophage activation actually in-

TABLE 11-3. Mediators of Effector Cell Activation

Cytokine	Number of Genes	Polypeptide Size	Cell Source	Cell Target	Primary Effects on Each Target
Gamma interferon	1	21-24 kD (homodimer)	T cell, NK cell	Mononuclear phagocyte	Activation
				Endothelial cell	Activation
				NK cell	Activation
				All	Increased class I and class II MHC molecules
Lymphotoxin	1	24 kD (homotrimer)	T cell	Neutrophil	Activation
				Endothelial cell	Activation
Interleukin-5	1	20 kD (monomer)	T cell	Eosinophil	Activation
				B cell	Growth and activation
Migration inhibition factor	?	?	T cell	Mononuclear phagocyte	Conversion from motile to immotile state

Abbreviations: NK, natural killer; kD, kilodalton; MHC, major histocompatibility complex.

volves several different responses, and macrophages are said to be activated when they perform a particular function being assayed. For example, IFN-γ fully activates macrophages to kill phagocytosed microbes but only partly activates macrophages to kill tumor cells.

2. *IFN-γ increases class I MHC molecule expression and, in contrast to type I IFN, also causes a wide variety of cell types to express class II MHC molecules.* Thus, IFN-γ amplifies the cognitive phase of the immune response by promoting the activation of class II-restricted CD4+ helper T cells. *In vivo,* IFN-γ can enhance both cellular and humoral immune responses through these actions at the cognitive phase.

3. *IFN-γ acts directly on T and B lymphocytes to promote differentiation.* It is one of the factors that promotes CTL maturation and also stimulates B cell secretion of antibody. In mice, it causes B cell switching to the Ig2a isotype. IFN-γ is not a growth factor for lymphocytes and often inhibits proliferation of lymphocytes, particularly B cells. In mice, IFN-γ can antagonize IL-4 mediated effects, such as isotype switching to IgE.

4. *IFN-γ activates neutrophils,* upregulating their respiratory burst. It is a less potent activator of neutrophils than TNF or lymphotoxin.

5. *IFN-γ is a potent activator of NK cells,* more so than type I IFN.

6. *IFN-γ is an activator of vascular endothelial cells,* promoting CD4+ T lymphocyte adhesion and morphologic alterations that facilitate lymphocyte extravasation. As mentioned earlier, IFN-γ also potentiates many of the actions of TNF on endothelial cells.

LYMPHOTOXIN

Lymphotoxin is a 21 to 24 kD glycoprotein that is approximately 30 per cent homologous to TNF and competes with TNF for binding to the same cell surface receptors. In humans, LT and TNF genes are located in tandem within the MHC on chromosome 6 (see Chapter 5). LT is produced exclusively by activated T lymphocytes and is often produced coordinately with IFN-γ by such cells. Human LT, unlike TNF, contains one or two N-linked oligosaccharides (accounting for the variability in molecular sizes). In further contrast to TNF, LT is synthesized as a true secretory protein without a membrane-spanning region.

Most studies have found little difference between the biologic effects of TNF and LT, consistent with their binding to the same receptor. The most important distinction between these cytokines appears to be that LT is exclusively synthesized by T cells, whereas TNF, although made by T cells, is predominantly derived from mononuclear phagocytes. In general, the quantities of LT synthesized by T cells are much less than the amounts of TNF made by LPS-stimulated mononuclear phagocytes and LT is not readily detected in the circulation. Therefore, LT is usually a locally acting paracrine factor and not a mediator of systemic injury. Although neither TNF nor LT is toxic for normal (non-neoplastic) cells, both factors may contribute to CTL-mediated lysis of target cells (see Chapter 12). Like TNF, LT is a potent activator of neutrophils and thus provides lymphocytes with a means of regulating acute inflammatory reactions. It is more potent than IFN-γ as an activator of neutrophils and the actions of LT are enhanced by IFN-γ. LT is also an activator of vascular endothelial cells, causing increased leukocyte adhesion, cytokine production, and morphologic changes that facilitate leukocyte extravasation. These effects, like those of TNF, are also enhanced by IFN-γ.

INTERLEUKIN-5

Interleukin-5 (IL-5) is a cytokine of approximately 20 kD produced by certain activated CD4+ T cells and by activated mast cells. IL-5 acts as a costimulator for the growth of antigen-activated mouse B cells and was previously called either B cell growth factor 2 or T cell replacing factor. IL-5 may function synergistically with other cytokines, such as IL-2 and IL-4, to stimulate the growth and differentiation of B cells. IL-5 has also been found to act on more mature B cells to cause increased synthesis of immunoglobulin, especially of IgA. These actions are discussed in greater detail in Chapter 9.

An important action of IL-5 is its ability to stimulate the growth and differentiation of eosinophils and to activate mature eosinophils in such a way that they can kill helminths. In mice, neutralizing antibodies to IL-5 inhibit the eosinophilia seen in response to helminthic infection. Thus, IL-5 provides a means by which T cells can regulate eosinophil-mediated inflammation.

MIGRATION INHIBITION FACTOR

We conclude our discussion of cytokines that regulate effector cells by considering the issue of migration inhibitor factor (MIF). One early view of cell mediated immune reactions proposed that mononuclear phagocyte accumulation in tissues depended on the retention of such cells in response to locally produced cytokines that inhibit motility. It now seems more likely that retention of leukocytes in the tissues is controlled primarily by expression of specific receptors for extracellular matrix molecules, such as integrins (see Box 7-4, Chapter 7). Nevertheless, one of the first cytokine activities identified was one that inhibited macrophage motility *in vitro,* called migration inhibition factor. MIF has still not been identified as a unique cytokine, although some recently cloned molecules appear to demonstrate MIF activity. At present, both the biochemical identity and biologic signifiance of MIF remain largely undefined.

Cytokines That Stimulate Hematopoiesis

Several of the cytokines generated during both natural immunity and antigen-induced specific immune responses have potent stimulatory effects on the growth and differentiation of bone marrow pro-

CELLULAR AND MOLECULAR IMMUNOLOGY

ABUL K. ABBAS, M.B.B.S.
Professor of Pathology
Harvard Medical School and Brigham and Women's Hospital
Boston, Massachusetts

ANDREW H. LICHTMAN, M.D., Ph.D.
Assistant Professor of Pathology
Harvard Medical School and Brigham and Women's Hospital
Boston, Massachusetts

JORDAN S. POBER, M.D., Ph.D.
Professor of Pathology and Immunobiology
Yale University School of Medicine
New Haven, Connecticut

W.B. SAUNDERS COMPANY
Harcourt Brace Jovanovich, Inc.
Philadelphia London Toronto Montreal Sydney Tokyo

W. B. Saunders Company
Harcourt Brace Jovanovich, Inc.
The Curtis Center
Independence Square West
Philadelphia, PA 19106

Library of Congress Cataloging-in-Publication Data

Abbas, Abul K.
Cellular and molecular immunology / Abul K. Abbas, Andrew H. Lichtman, Jordan S. Pober.
p. cm.
ISBN 0-7216-3032-4
1. Cellular immunity. 2. Immunity—Molecular aspects. I. Lichtman, Andrew H. II. Pober, Jordan S. III. Title.
[DNLM: 1. Immunity, Cellular. 2. Lymphocytes—immunology. QW 568 A122c]
QR185.5.A23 1991
616.07'9—dc20
DNLM/DLC

Editor: Martin J. Wonsiewicz
Designer: Paul M. Fry
Production Manager: Peter Faber
Manuscript Editor: Carol Robins
Illustrator: Risa Clow
Illustration Coordinator: Brett MacNaughton
Indexer: Linda Van Pelt

ISBN 0-7216-3324-2

Cellular and Molecular Immunology

Printed in the United States of America.

Last digit is the print number: 9 8 7 6 5 4

CHAPTER TWO

CELLS AND TISSUES OF THE IMMUNE SYSTEM

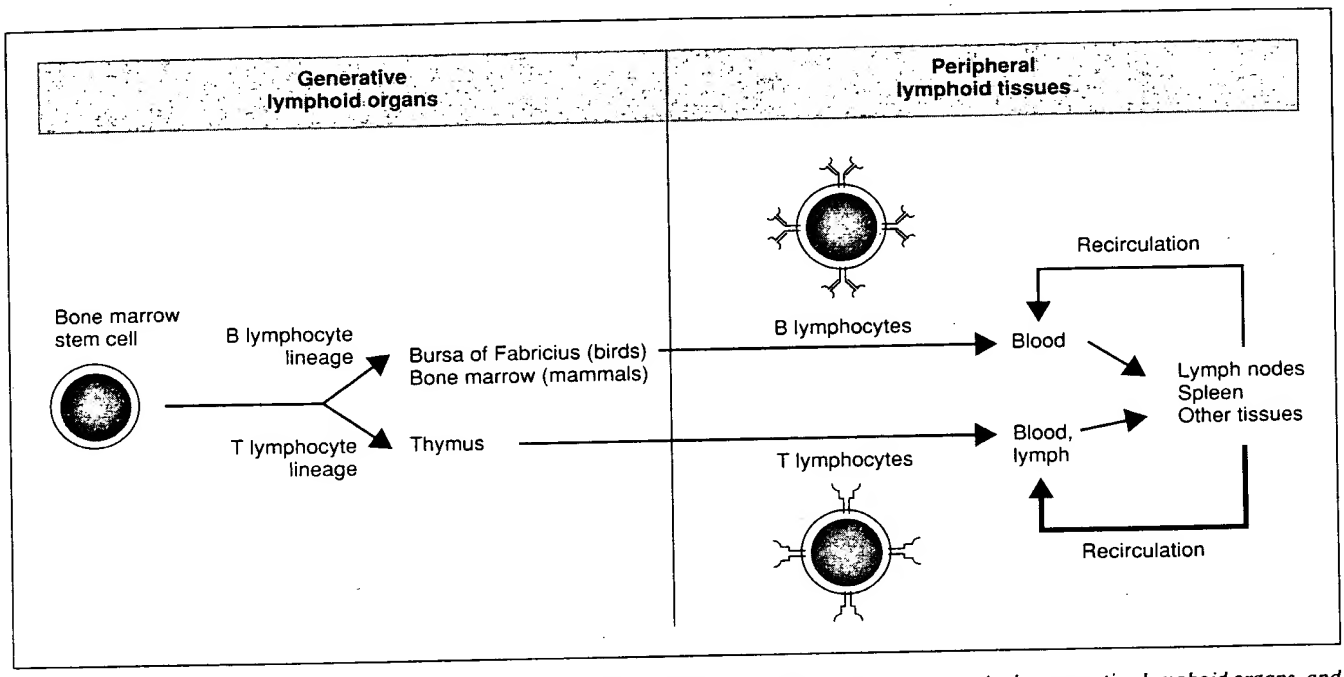

FIGURE 2-7. Maturation of lymphocytes. *Development of mature lymphocytes prior to antigen exposure occurs in the generative lymphoid organs, and immune responses to foreign antigens occur in the peripheral lymphoid tissues.*

brae, iliac bones, and ribs. The red marrow that is found in these bones consists of a sponge-like reticular framework located between bony trabeculae. The spaces in this framework are filled by fat cells and the precursors of blood cells, which mature and exit via the dense network of vascular sinuses to become part of the circulatory system.

All the blood cells originate from a common **stem cell** that becomes committed to differentiate along particular lineages, i.e., erythroid, megakaryocytic, granulocytic, monocytic, and lymphocytic (Fig. 2-8). Cytokines are known to stimulate the proliferation and maturation of various precursors. Since these growth factors are assayed by their ability to stimulate different types of leukocyte colonies to develop from marrow cells *in vitro,* they are called "colony-stimulating factors" (CSFs). Several of these cytokines are produced by T lymphocytes, including interleukin-3 (IL-3, also called multi-CSF), which acts on all stem cells, and granulocyte-monocyte-CSF (GM-CSF), which stimulates the formation of granulocytes and monocytes. Macrophages and marrow stromal cells produce GM-CSF and additional CSFs specific for granulocytes (G-CSF) or monocytes (M-CSF). Macrophages and stromal cells in the bone marrow produce two other cytokines, called interleukin-1 and interleukin-6, which further enhance colony formation by hematopoietic precursors in the presence of CSFs. A cytokine called interleukin-7, also produced by marrow stromal cells, has been shown to preferentially stimulate the maturation of B lymphocytes from marrow precursors. The properties and functions of these cytokines are described in Chapter 11. However, little is known about the nature of the uncommitted stem cell or the mechanisms that regulate its commitment to specific lineages. In 1988, techniques for reconstituting the immune system of congenitally immunodeficient mice with human lymphohematopoietic stem cells were described. These immunodeficient mice lack T and B lymphocytes, and after the implantation of human hematopoietic tissues, mature human T and B cells develop in the animals and populate the circulation and peripheral lymphoid tissues. Such approaches hold great promise for more precise identification and characterization of stem cells and their developmental pathways.

Thymus

The thymus is a bilobed organ situated in the anterior mediastinum. Each lobe is divided into multiple lobules by fibrous septa, and each lobule consists of an outer cortex and an inner medulla (Fig. 2-9). The cortex contains a dense collection of T lymphocytes, and the lighter-staining medulla is more sparsely populated with lymphocytes. Scattered throughout the thymus are non-lymphoid epithelial cells, which have abundant cytoplasm, as well as bone marrow-derived dendritic cells and macrophages. In the medulla are structures called **Hassall's corpuscles,** which are composed of tightly packed whorls of epithelial cells that may be remnants of degenerating cells. The thymus has a rich vascular supply and efferent lymphatic vessels that drain into mediastinal lymph nodes.

The lymphocytes in the thymus, also called **thymocytes,** are T lymphocytes at various stages of matu-

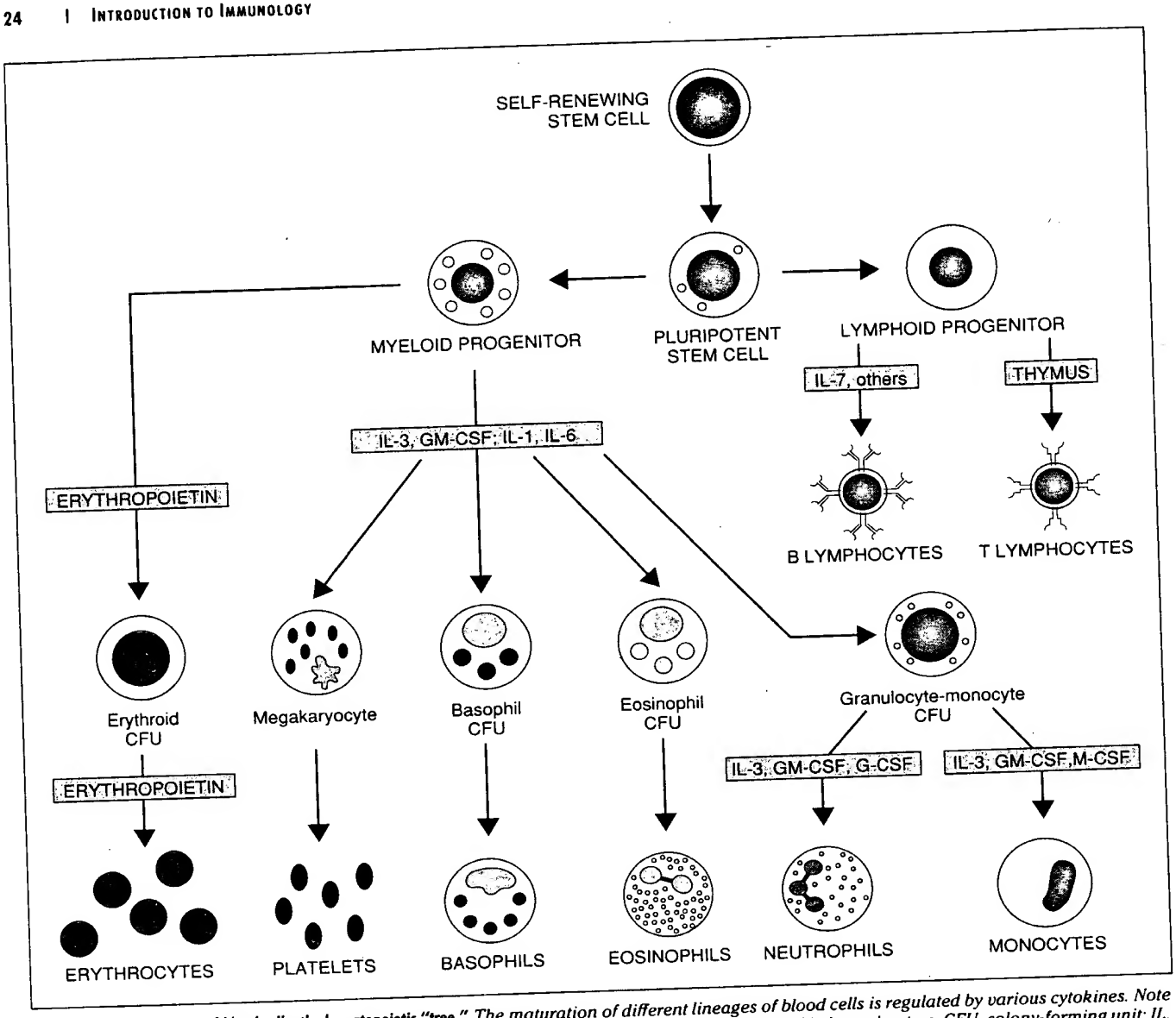

FIGURE 2 – 8. Maturation of blood cells: the hematopoietic "tree." *The maturation of different lineages of blood cells is regulated by various cytokines. Note that the maturation of T and B lymphocytes is illustrated in summary form and is discussed in detail in later chapters. CFU, colony-forming unit; IL, interleukin; GM - CSF, granulocyte-macrophage colony-stimulating factor.*

ration. Precursors that are committed to the T cell lineage enter the thymic cortex via blood vessels. It is not known whether B cell precursors enter the thymus and fail to survive or whether there are mechanisms that ensure that only cells committed to developing into T lymphocytes can enter the thymus. The most immature thymocytes do not express receptors for antigens or surface markers, including CD4 and CD8, that are characteristic of the mature phenotype. These immature cells migrate from the cortex toward the medulla and come into contact with epithelial cells, macrophages, and dendritic cells. Efficient contact may occur in lymphoepithelial complexes in which lymphocytes are found closely apposed to the invaginated plasma membranes of large epithelial cells called **"nurse cells."** En route to the medulla, thymocytes begin to express receptors for antigens and surface markers that are present on mature, peripheral T lymphocytes. Thus, the medulla contains mostly mature T cells, and only mature $CD4^+$ or $CD8^+$ T cells exit the thymus and enter the blood, lymph, and peripheral lymphoid tissues.

From the large number of primitive T cells that enter the thymus, cells that might recognize self antigens do not survive, whereas cells whose receptors are specific for foreign antigens are stimulated to mature. These selection processes, which are critical for the ability of the immune system to discriminate between self and non-self, are described in considerable detail in Chapter 8. The thymus is the site of remarkable proliferation as well as elimination of lymphocytes, which presumably reflect the selection of foreign antigen-specific cells and the deletion of potentially self-reactive T cells. It is estimated that in

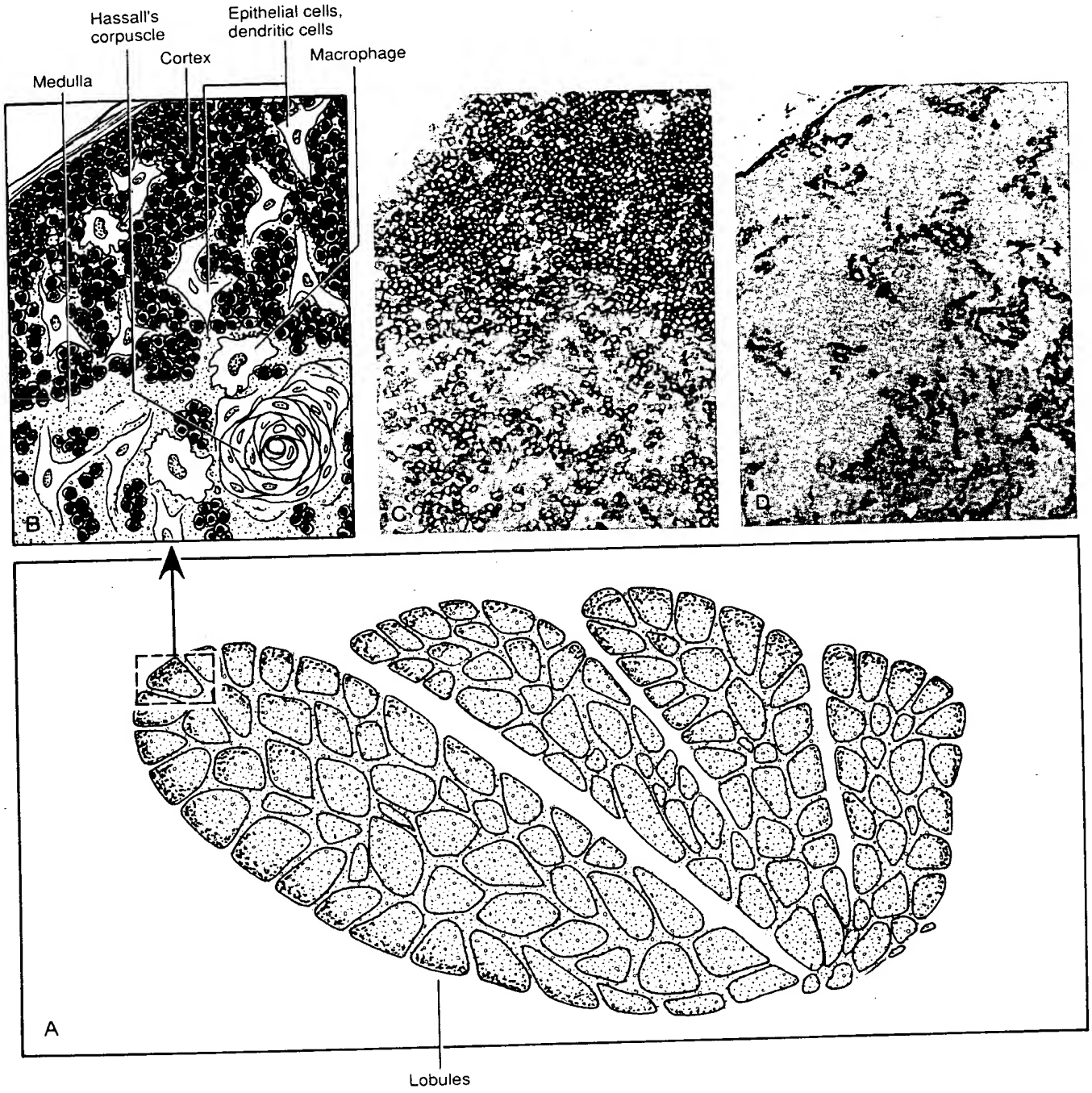

FIGURE 2 – 9. Morphology of the thymus.

A. *Schematic diagram of the thymus, illustrating two main lobes (the one on the right being subdivided into smaller lobes in this thymus), composed of multiple lobules.*

B. *Diagram of the edge of a lobule showing the cells of the cortex and medulla.*

C. *T lymphocytes in the cortex of the thymus, detected by an immunoperoxidase stain with an antibody specific for T cells. (Immunoperoxidase staining is described in Chapter 3; positive cells appear dark.) (Courtesy of Dr. G. S. Pinkus, Department of Pathology, Brigham and Women's Hospital, Boston.)*

D. *Epithelial cells scattered throughout the cortex and medulla, detected by an immunoperoxidase stain specific for keratin, an intracellular intermediate filament protein. (Courtesy of Dr. G. S. Pinkus, Department of Pathology, Brigham and Women's Hospital, Boston.)*

mice, about 50×10^6 immature cells enter the thymus each day and fewer than 1×10^6 mature cells leave. The thymus undergoes physiologic involution with aging, so that by puberty it is difficult to locate. Maturation and selection of T lymphocytes continue well into adult life, suggesting that either the remnants of the thymus that are present in adults are adequate for performing these functions or extrathymic T cell maturation can also occur. However, no such extrathymic sites of T cell development have been identified.

Lymph Nodes

Lymph nodes are small nodular aggregates of lymphoid tissue situated along lymphatic channels throughout the body. Epithelia, such as the skin and the mucosa of the gastrointestinal and respiratory tracts, as well as connective tissues and most organs have a lymphatic drainage. Antigens that enter through any of these portals end up in lymphatic vessels whose contents are "sampled" by lymph nodes for the presence of foreign substances. Each node is surrounded by a fibrous capsule that is pierced by numerous afferent lymphatics, which empty the lymph into a subcapsular sinus (Fig. 2-10). The node consists of an outer cortex in which there are aggregates of cells constituting the **follicles,** some of which contain central areas called **germinal centers** that stain lightly with commonly used histologic stains. The inner medulla contains less dense lymphocytes and mononuclear phagocytes scattered among lymphatic and vascular sinusoids. Lymphocytes and accessory cells are often found in close proximity but do not form intercellular junctions, which is probably important for maintaining their ability to migrate and recirculate between the lymph, blood, and tissues. The lymph that enters the subcapsular sinus percolates through the cortex and medulla and exits via a single efferent lymphatic located in the hilum of the node. In addition, each node has a vascular supply with afferent and efferent vessels at the hilum.

Different classes of lymphocytes and non-lymphoid accessory cells are sequestered in particular areas of the node. Follicles without germinal centers, which are called **primary follicles,** contain predominantly mature, resting B lymphocytes that have apparently not been stimulated recently by antigens. The germinal centers, which develop in response to antigenic stimulation, contain numerous large lymphocytes with phenotypic characteristics of activated B cells. The germinal centers are believed to be one of the sites where B lymphocytes proliferate and differentiate into antibody-secreting cells in response to antigenic stimulation. It is estimated that the cell cycle time of germinal center B cells is as short as 6 hours. Since plasma cells are rare at these sites, it is possible that the terminal differentiation of B cells occurs outside the germinal centers. It is also believed that the activation of memory B cells, the production of antibodies with increased affinities for antigens, and the appearance of different classes of antibodies (described in Chapters 4 and 9) are three aspects of antigen-stimulated B cell differentiation that are initiated largely in germinal centers. Since these events usually require the participation of helper T lymphocytes, it is not surprising that germinal center development is T cell-dependent and is not seen in individuals congenitally deficient in T cells. In addition to lymphocytes, lymphoid follicles contain macrophages and scattered **dendritic cells** (also called interdigitating reticular cells) that function as accessory cells in immune responses. Also, in the germinal centers there are **follicular dendritic cells** that have long cytoplasmic processes, express large numbers of receptors for antibodies (Fc receptors) on their surfaces, and are different from the "dendritic cells" mentioned above. Follicular dendritic cells are believed to be important in capturing antigens complexed with preformed antibodies and, therefore, in the activation of memory B cells to generate secondary antibody responses.

The T lymphocytes are located predominantly in the interfollicular areas of the cortex and paracortical zones in the medulla (Fig. 2-10). Most of these are $CD4^+$ helper T cells, intermingled with relatively sparse $CD8^+$ cells. Some $CD4^+$ T cells are also scattered in germinal centers, where their role may be to help the proliferation and differentiation of antigen-stimulated B lymphocytes. The proximity of helper T cells and the B cells that are the recipients of T cell help is important because helper function is mediated largely by secreted cytokines, which act at short distances, close to the sites where they are produced (see Chapter 9). Dendritic (interdigitating reticular) cells are abundant in the T cell areas, which is consistent with their postulated role in presenting foreign antigens to T lymphocytes.

The medulla contains scattered lymphocytes, large numbers of macrophages and dendritic cells, and, in nodes draining sites of immunization, numerous plasma cells, all of which are interspersed with lymphatic channels.

The mechanisms responsible for the anatomic sequestration of different classes of lymphocytes in distinct areas of the node are unclear. One possibility is that compartmentalization is maintained by specific adhesions of different lymphocytes with stromal cells or extracellular matrix proteins. Although the anatomy of the immune response is poorly understood, it is likely that this cellular organization promotes interactions between the participating cell types and is critically important for the generation of immunity. Studies using labeled antigens indicate that a protein antigen that enters the lymph node in an unimmunized individual is trapped by macrophages and dendritic cells and largely degraded. Peptide fragments of the injected antigen, attached to the surfaces of accessory cells, stimulate helper T lymphocytes (see Chapter 6). The first wave of mitotic activity is seen in the T cell zones within 1 to 2 days after immunization. Proliferation of B cells follows, after which germinal centers develop and the B lympho-

The Developmentally Regulated Expression of *Twisted Gastrulation* Reveals a Role for Bone Morphogenetic Proteins in the Control of T Cell Development

Daniel Graf,[1,3] Suran Nethisinghe,[1] Donald B. Palmer,[2] Amanda G. Fisher,[1] and Matthias Merkenschlager[1]

[1]*Lymphocyte Development Group, Medical Research Council Clinical Sciences Centre, and* [2]*Department of Immunology, Imperial College of Medicine, London W12 0NN, United Kingdom*
[3]*Institute of Immunology Biomedical Sciences Research Centre 'Al Fleming', 166 72 Vari, Greece*

Abstract

The evolutionarily conserved, secreted protein Twisted gastrulation (Tsg) modulates morphogenetic effects of decapentaplegic (dpp) and its orthologs, the bone morphogenetic proteins 2 and 4 (BMP2/4), in early *Drosophila* and vertebrate embryos. We have uncovered a role for Tsg at a much later stage of mammalian development, during T cell differentiation in the thymus. BMP4 is expressed by thymic stroma and inhibits the proliferation of $CD4^-CD8^-$ double-negative (DN) thymocytes and their differentiation to the $CD4^+CD8^+$ double-positive (DP) stage in vitro. *Tsg* is expressed by thymocytes and up-regulated after T cell receptor signaling at two developmental checkpoints, the transition from the DN to the DP and from the DP to the $CD4^+$ or $CD8^+$ single-positive stage. Tsg can synergize with the BMP inhibitor chordin to block the BMP4-mediated inhibition of thymocyte proliferation and differentiation. These data suggest that the developmentally regulated expression of *Tsg* may allow thymocytes to temporarily withdraw from inhibitory BMP signals.

Key words: BMP4 • Twisted gastrulation • thymocyte development • chordin • morphogen

Introduction

Thymocyte differentiation is controlled by signals via the pre-TCR and the TCR (for reviews, see references 1–3). Progression from the $CD4^-CD8^-$ double-negative (DN)* to the $CD4^+CD8^+$ double-positive (DP) stage in cells destined for the TCR αβ lineage is driven by pre-TCR signaling and requires TCRβ chain rearrangement, expression, and pairing with the pre-TCR α chain. The acquisition of CD4 and CD8 is accompanied by a burst of proliferation, the cessation of further V to DJ rearrangement at the TCR β locus and proceeds through a transitional stage where (in the mouse strains used in this study) CD8 is expressed before CD4 (1). Recombination-deficient thymocytes are blocked at the $CD4^-CD8^-CD25^+CD44^-$ stage of development but antibodies to the CD3ε signaling chain can mimic pre-TCR signaling and trigger progression to the DP stage (2). Further development from the DP to the $CD4^+$ or $CD8^+$ single-positive (SP) stage of development requires rearrangement and pairing of TCRα with TCRβ and the engagement of the resulting heterodimer with suitable peptide/MHC ligands on thymic stromal cells (3).

The control of cellular differentiation by antigen receptor rearrangement, expression, and engagement is unique to lymphocytes and (in evolutionary terms) a recent addition to older systems comprising soluble and cell-associated factors that control the survival, growth, and differentiation of cells and tissues in all multicellular organisms. For example, thymocyte survival and differentiation are promoted by cytokines including IL-7 (4), c-Kit ligand (5), IL-1, and TNF (6), and signaling through Notch (7) participates in T cell lineage commitment (8). Comparatively little is known about the role of patterning molecules or morphogens in lymphocyte development (9–12).

In a screen for genes that are regulated in a developmental stage-specific manner in thymocytes (13) we have identified *Twisted gastrulation* (*Tsg*; reference 14). Recent studies have revealed that the Tsg protein interacts with *Drosophila*

Address correspondence to M. Merkenschlager, Lymphocyte Development Group, Imperial College of Medicine, Hammersmith Campus, Du Cane Rd., London W12 0NN, UK. Phone: 44-208-383-8236; Fax: 44-208-383-8338; E-mail: matthias.merkenschlager@csc.mrc.ac.uk

**Abbreviations used in this paper:* BMP, bone morphogenetic protein; DN, double negative; DP, double positive; dpp, decapentaplegic; pSmad, phospho-Smad; Rag, recombination activating gene; RT, reverse transcription; sog, short gastrulation; SP, single positive; Tsg, twisted gastrulation.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/2002/07/163/9 $5.00
Volume 196, Number 2, July 15, 2002 163–171
http://www.jem.org/cgi/doi/10.1084/jem.20020276

decapentaplegic (dpp), the vertebrate dpp orthologs bone morphogenetic protein (BMP)2/4, and also the extracellular dpp/BMP inhibitors short gastrulation (sog)/chordin (15–20). In addition, Tsg can alter the proteolytic processing of sog/chordin by extracellular metalloproteases (16, 20). As a result, Tsg affects the binding of dpp/BMP2/4 to their cellular receptors and downstream signaling events mediated by the phosphorylation, nuclear translocation, and transcriptional activity of Smad proteins (for a review, see reference 21) positively (15, 20) or negatively (16–20). BMPs belong to a family of secreted signaling molecules the founding member of which, TGFβ, is essential for immune homeostasis (11, 22). In addition to a well-established role in embryonic patterning and development (23), BMP4 has been linked to hematopoesis: it specifies ventral mesoderm and blood cell formation in the *Xenopus* embryo (24), cooperates with VEGF to enhance hematopoetic cell generation from ES cells (25), is expressed in the human fetal AGM region (26), and regulates primitive human hematopoetic cell proliferation (27). There is evidence that components of the BMP signaling pathway are expressed in the thymus, including BMP4 itself (9), the extracellular BMP inhibitor chordin (28), the BMP receptor components activin-like kinase (ALK)-3 and -6 (BMPR1A and -B; reference 29), and Smad proteins (30, 31), the downstream mediators of BMP signaling. However, a role for BMP signals in thymocyte development has not been described. Furthermore, our understanding of *Tsg* is currently limited to early embryonic development (15–20, 32). We have investigated the effects of BMP4 and its modulation by Tsg during the transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage of thymocyte development.

Materials and Methods

Mouse Strains, Cell Sorting, Cell and Organ Culture. Thymi were derived from wild-type (BALB/c or C57BL/6), recombination activating gene (Rag)$1^{o/o}$ (33), or $A\beta^{o/o}$ $\beta2m^{o/o}$ (34, 35; referred to as $MHC^{o/o}$ in this manuscript). Where indicated, 3–4-wk-old Rag-$1^{o/o}$ mice were injected with 50 μg of the CD3ε mAb 2C11 (BD PharMingen). Thymocyte organ cultures and suspension cultures of mechanically dissociated or trypsinized fetal thymi were set up as hanging drops in inverted Terasaki plates (Nunc) in serum-free AIM-V lymphocyte medium (GIBCO BRL) supplemented with 2×10^{-5} M 2-ME, where indicated in the presence of recombinant BMP2, -4, and -7, chordin, neutralizing anti-BMP4 or BMPR-IA/Fc (all from R&D Systems), TGFβ1 (Sigma-Aldrich), or the CD3ε antibody 2C11 (BD PharMingen). Recombinant mouse Tsg was produced in X63 myeloma cells transfected with full-length mTsg cDNA (14) tagged with a COOH-terminal HA epitope and inserted into the BCMGS neo vector (36). Supernatant was concentrated, mTsg-HA captured with anti-HA–conjugated agarose beads (Sigma-Aldrich) and eluted by incubation of the washed beads with 100 μg/ml HA peptide (Sigma-Aldrich). Western analysis of the material with an anti-HA antibody (Sigma-Aldrich) showed a single band at ~25 kD. Tsg concentration was estimated by gel staining since the HA peptide used for elution interfered with measurement of protein concentration. Supernatant from X63 cells transfected with empty BCMGS neo vector treated in the same way served as a control for mTsg-HA. All Tsg effects were confirmed using commercial mTsg (R&D Systems) which became available during the course of this study.

For some experiments thymocytes were stained with biotinylated antibodies to CD4 or CD8 and depleted with streptavidin-coated paramagnetic beads (Dynal). Thymic stromal cells were prepared by trypsinization of deoxyguanosine-treated fetal thymi and centrifugation over 55% Percoll (Amersham Pharmacia Biotech). Wild-type thymi were cultured for 18 to 72 h, thymus cell suspensions and thymocytes for 18 h, and Rag$1^{o/o}$ thymi for 48–72 h in the presence of the 2C11 mAb at 1 μg/ml. For phenotypic analysis on a FACSCalibur™ (Becton Dickinson) cells were counted and stained with CD4-PE or Cy-5 and CD8-FITC or -Cy5 (Caltag Laboratories), fixed for 10 min at room temperature in 0.2% paraformaldehyde, permeabilized with 0.2% Tween-20 for 15 min at 37°C, and incubated with 7-amino actinomycin D (7AAD). For sorting of DN and CD8 transitional cells on a FACS Vantage™ (Becton Dickinson), thymocytes were stained with CD4-Tricolor, CD8-PE (Caltag Laboratories), and heat stable antigen (HSA)-FITC.

Immunohistochemistry and RNA In Situ Hybridization on Frozen Sections. Thymi from 3–4-wk-old C57BL/6 mice were snap frozen, 6-μm sections were prepared. For immunohistochemistry, sections were fixed in acetone and stained using polyclonal goat anti-BMP2/4 (R&D Systems) followed by horseradish peroxidase–conjugated rabbit anti–goat Ig antibody (Dako) and liquid DAB substrate-chromogen solution (Dako) according to the manufacturer's instructions. Slides were counterstained with Mayer's Hematoxylin (Sigma-Aldrich) and mounted in Kaiser's solution (14). Sense and antisense riboprobes were synthesized from a cloned PCR fragment spanning exon 4 of mTsg (14) in the presence of 11-dUTP digoxigenin (Roche) with T3 or T7 RNA polymerase. After hybridization, high-stringency washing, and RNase A digestion, the sections were incubated with alkaline phosphatase–conjugated anti-digoxigenin (Roche). BCIP/NBT (Roche) was used as substrate for detection. The sections were counterstained with nuclear fast red (Vector Laboratories), mounted in Mowiol solution (Calbiochem), and photographed.

Molecular Cloning and Analysis. Expression studies were performed as reported previously (14, 37). For Northern blots, 1–5 μg total RNA were separated on 1% agarose gels containing formamide. The RNA was transferred to HybondN+ Nylon membranes and hybridized in 1.5× SSPE, 10% PEG 8000, 7% SDS at 68°C overnight. After washing in decreasing SSC concentrations and a final stringency wash in 0.1× SSC, 0.5% SDS at 60°C the membranes were exposed to BioMax-MS film (Eastman Kodak Co.).

For reverse transcription (RT)-PCR experiments, 0.3–1 μg total RNA were reverse transcribed with AMV reverse transcriptase (Promega) and amplified with the following primers at annealing temperatures of 60°C to 63°C: BMP2 no. 465 TACCGCAGGCACTCAGG, no. 466 CATTCCACCCCACATCACT; BMP4 no. 467 CGAGGCGACACTTCTACAG, no. 468 TGGGGGCTTCATAACCT; chordin: no. 473 CAAGCCTCAGCGGAAGAA, no. 474 CAAGCCCAGCCAATAGAACT; GAPDH: no. 251 TCTTCTTGTGCAGTGCC, no. 252 ACTCCACGACATACTCAGC. PCR products were analyzed on 2% agarose gels and the identity of the bands was confirmed by blotting and hybridizing with specific probes.

Western Blotting. Phospho-Smad (pSmad)-2–specific antibodies were used to assess the phosphorylation status of Smad-2. Thymocyte lysates were separated (2×10^6 cell equivalents per lane) on 10% SDS polyacrylamide gels and blotted onto PVDF

membranes (NEN Life Science Products). Blots were blocked (1% nonfat milk in PBS, 0.1% Tween 20 for p-Smad-2; 5% nonfat milk in PBS) and probed with rabbit anti-pSmad2 (Upstate Biotechnology 06–829) followed by alkaline phosphatase–conjugated anti–rabbit IgG and enhanced chemoluminescence detection (Western Star; Tropix) on MR-1 film (Eastman Kodak Co.). Blots were stripped and reprobed for total Smad-2 and subsequently for lamin B to control for equal loading and transfer efficiency (not shown).

Results

Tsg Up-regulation After TCR Signaling at Control Points in Thymocyte Development. We undertook a screen to find novel genes involved in T cell development. MHC-naive $CD4^+CD8^+$ DP thymocytes (isolated from $MHC^{o/o}$ mice) were cocultured with thymic stromal cells derived from MHC-expressing (wild-type) or, as a control, from $MHC^{o/o}$ thymi (37). This system allowed a comparison of gene expression profiles between MHC-exposed and MHC-naive thymocytes by differential display PCR (13). The characterization of a band consistently up-regulated upon TCR signaling resulted in the cloning of the mouse homologue of *Tsg* (14). Northern blotting confirmed increased *Tsg* expression by MHC-naive DP thymocytes between 6 and 24 h after treatment with antibodies to the TCR alone or in combination with anti-CD28 (Fig. 1 a). To investigate *Tsg* expression at the pre-TCR–driven transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage we injected $Rag1^{o/o}$ mice with antibodies to CD3ε. Thymocyte development in $Rag1^{o/o}$ mice is blocked at the $CD44^-CD25^+$ DN stage of differentiation and can be rescued by CD3ε treatment in vivo which triggers a synchronous wave of differentiation from the $CD44^-CD25^+$ DN to the DP stage (2). This was accompanied by a rise in *Tsg* expression that occurred around 48 h after anti-CD3 injection (Fig. 1 b), at a time when thymocytes showed reduced CD25 expression and increased proliferation but were still DN (reference 2, and data not shown). We conclude that *Tsg* is differentially expressed after TCR signaling at two developmental checkpoints, the transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP and from the DP to the $CD4^+$ or $CD8^+$ SP stage.

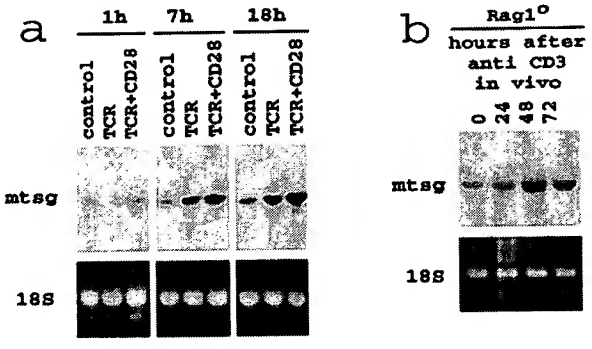

Figure 1. TCR-regulated Tsg expression by thymocytes. (a) Increased *Tsg* mRNA expression by MHC-naive DP thymocytes in response to antibodies to the TCR, with or without CD28 engagement. (b) Increased *Tsg* mRNA expression by DN thymocytes 48 h after injection of $Rag\text{-}1^{o/o}$ mice with anti-CD3ε. In additional experiments, *Tsg* upregulation occurred between 24 and 72 h and persisted until 120 h after injection.

The recognized role of Tsg as a modulator of BMP signaling (15–20) prompted us to reevaluate the expression of components of the BMP signaling pathway in the thymus (see Introduction). RT-PCR showed the expression of BMP2, BMP4, BMP7, and chordin in total thymus tissue (Fig. 2 a). Analysis of separated thymocytes and thymic stroma established thymic stromal cells as the source of BMP2/4 and chordin. BMP7 was expressed by both thymocytes and stromal cells (Fig. 2 a). Semiquantitative RT-PCR analysis confirmed that thymocytes expressed BMP7 at levels similar to total thymus whereas the levels of BMP4 and chordin expression in thymocytes were 20- to 100-fold lower in thymocytes than total thymus (data not shown). The distribution of BMP2/4 protein in the thymus was visualized by immunohistochemistry on frozen sections (Fig. 2, b–e). We noted marked regional differences with staining predominantly of subcapsular (sc) and medullary (med) regions. Within these areas the signal appeared 'patchy' rather than uniform (Fig. 2, c and e). In situ hybridization showed *Tsg* expressing cells distributed throughout the cortex and the medulla (Fig. 2, f–h).

BMP4 Can Interfere with Cell Cycle Activity and Developmental Progression of DN Thymocytes. To assess the functional effects of BMPs we established thymic organ cultures at embryonic day 15.5 (E15.5), a time in ontogeny when all thymocytes are DN, and analyzed the cell cycle distribution as well as the phenotype of thymocytes that developed in these cultures. Addition of BMP4 at 100 ng/ml (~6 nM) reduced the percentage of thymocytes in S and G2/M phase of the cell cycle by 42 ± 9% (Fig. 3, a and b). This inhibition was largely abrogated by a neutralizing antibody to BMP4 (Fig. 3 a). Conversely, treatment of thymic organ cultures with recombinant chordin 2 μg/ml (~20 nM) increased the percentage of thymocytes in S and G2/M by 59 ± 30% (Fig. 3 a). As chordin is an extracellular inhibitor specifically of BMPs but not of TGFβ or activin (21), the increased proliferation of thymocytes in intact thymic lobes treated with chordin suggests that thymocyte cell cycle activity is subject to BMP-mediated inhibition in situ.

In addition to proliferation, BMP4 treatment of E15.5 thymic organ cultures interfered with the developmental progression from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage (Fig. 3 b) and reduced the generation of DP cells on average by 52 ± 21% (Fig. 3 c). In control experiments, the survival of sorted E17 DP thymocytes was unaffected by overnight culture in BMP4 (100 ng/ml), indicating that BMP4 was not simply toxic to DP thymocytes (data not shown).

The reduced generation of DP thymocytes in the presence of BMP4 could be explained either by a delay in TCRβ chain rearrangement or by a reduced response to pre-TCR signaling. To address this issue we assayed BMP4

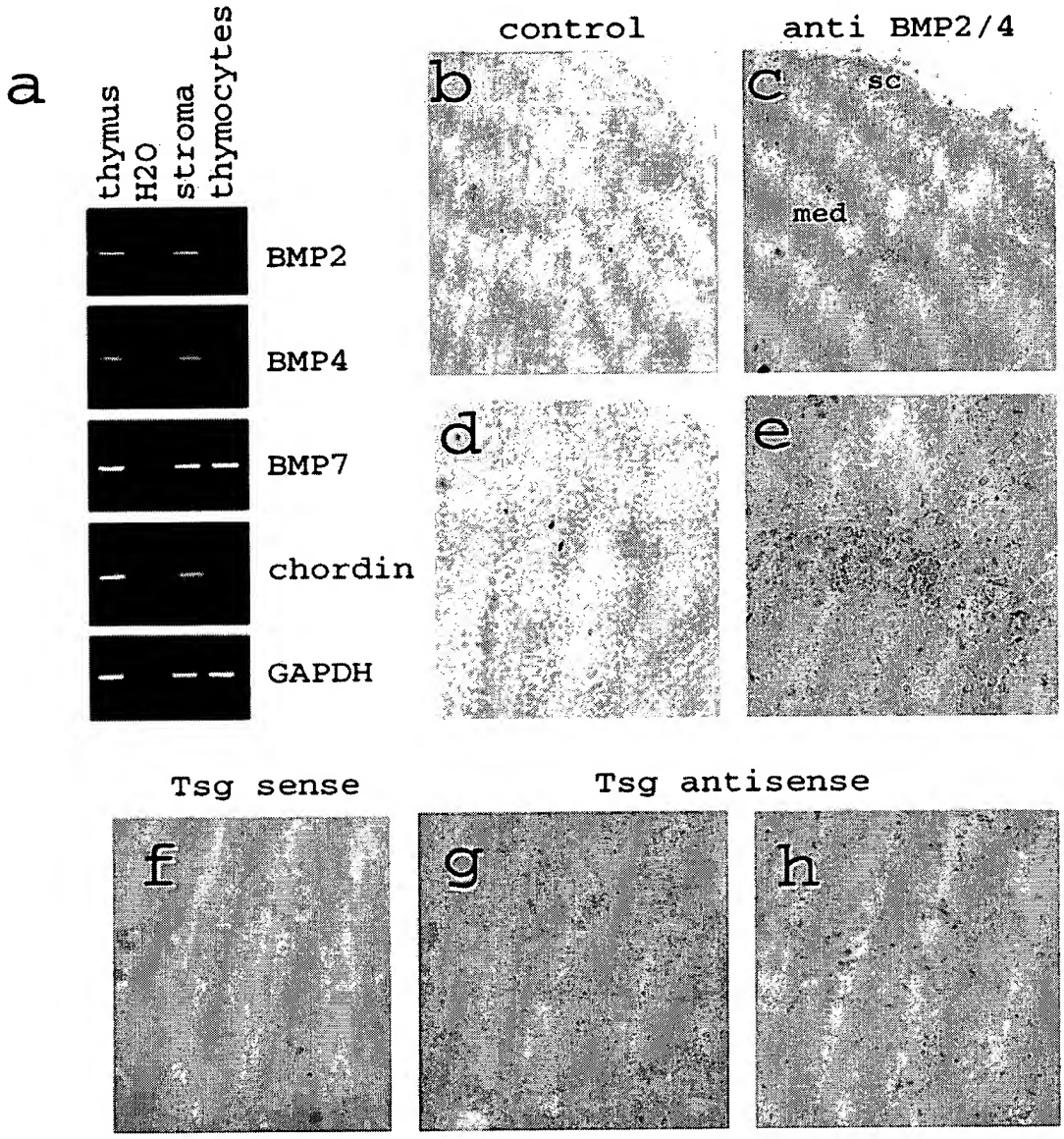

Figure 2. Expression and distribution of intrathymic BMPs and chordin. (a) Total thymus or isolated stromal cells and thymocytes were subjected to RT-PCR analysis to establish the sources of BMP2, BMP4, BMP7, and chordin in the thymus. GAPDH served as a positive control. H_2O lanes contained no cDNA. (b–e) Immunoperoxidase staining of normal 3–4-wk-old mouse (C57BL/6) thymus with goat anti-BMP/24 (c and e) or control goat antibody (b and d). BMP2/4 staining (brown) is seen mainly within supcapsular (sc) and medullary (med, outlined in c) regions (original magnification: ×200, b and c) and 'patchy' within positive areas (original magnification: ×400, d and e). (f–h) In situ hybridization of normal 3–4-wk-old mouse (C57BL/6) thymus with Tsg sense (f) and antisense (g and h) probes. *Tsg* expressing cells are distributed throughout the cortex and the medulla. Original magnification: ×100 (f and g), ×200 (h).

effects on the differentiation of Rag1-deficient thymocytes in response to CD3ε antibody (1 μg/ml) as a surrogate signal which does not require TCRβ chain rearrangement. BMP4 treatment reduced the development of DP thymocytes in Rag1$^{o/o}$ organ cultures treated with CD3ε antibodies by 46 ± 22% (Fig. 3 c).

To ask whether BMP4 inhibition of the DN to DP transition required an intact thymic microenvironment, E15.5 thymi were disrupted by trypsinization to yield suspensions containing all thymic cell types including thymic stroma. BMP4 blocked the DN to DP transition by 52 ± 17%. Similarly, BMP4 blocked the DN to DP transition in me-

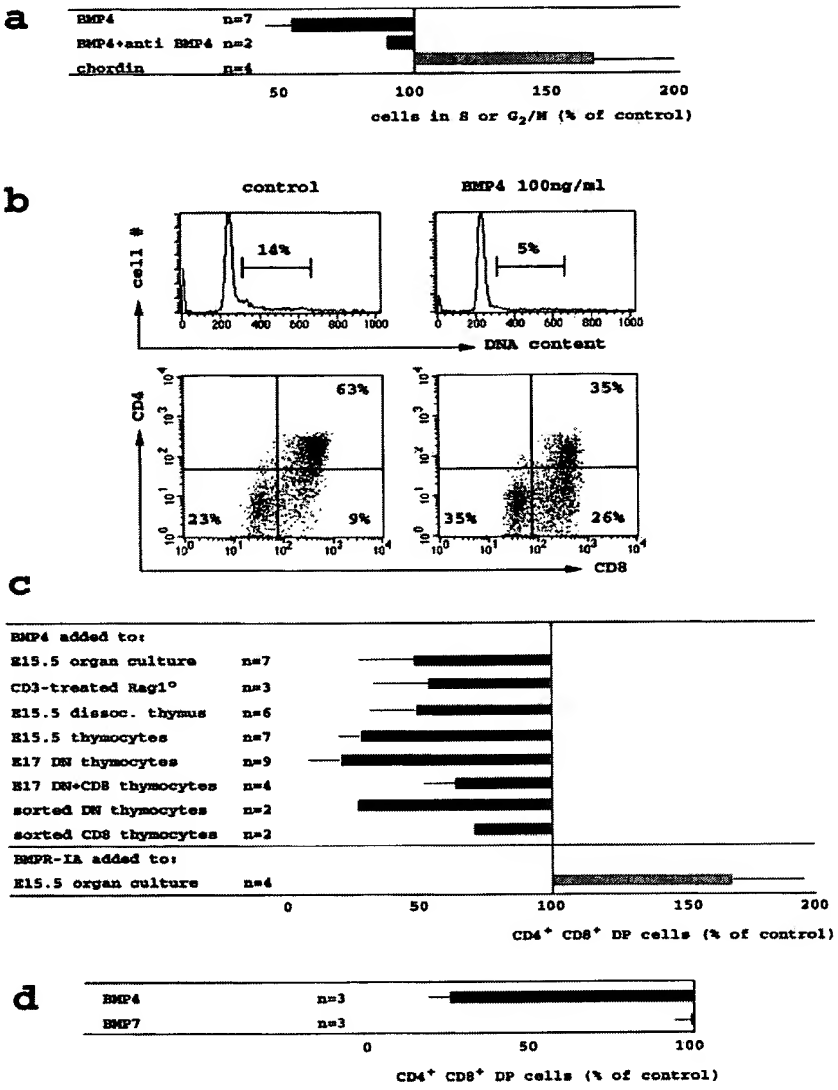

Figure 3. BMP4 inhibits the cell cycle activity and the developmental progression of DN thymocytes to the DP stage. (a) E15.5 thymic lobes were cultured in hanging drops for 24–36 h with BMP4 (100 ng/ml, 6 nM approximately), BMP4 (100 ng/ml) plus neutralizing anti-BMP4 (10 μg/ml) or chordin (2 μg/ml, 20 nM approximately). Thymocytes were stained for CD4 and CD8, fixed, permeabilized, and DNA content was visualized by 7AAD. The fraction of cells with a DNA content >G1 is shown relative to control cultures (16 ± 7% >G1). (b) Reduced cell cycle activity and impaired developmental progression in BMP4-treated E15.5 thymic organ cultures (36 h, methods as in a). (c) BMP4 (100 ng/ml) inhibited the DN to DP transition in E15.5 thymic organ cultures by 52 ± 21% (from 50 ± 10% DP to 25 ± 13% at 36 h, top row) and in $Rag1^{o/o}$ thymic organ cultures treated for 72 h with anti-CD3ε (1 μg/ml) by 46 ± 22% (from 57 ± 4% DP to 32 ± 12%, row two). BMP4 reduced the generation of DP cells in proteolytically dissociated E15.5 thymus suspensions by 52 ± 17% (from 59 ± 13% DP to 28 ± 9%, third row), in mechanically prepared E15.5 thymocyte suspensions by 72 ± 8% (from 44 ± 19% DP to 13 ± 6%, row four), in DN cells prepared by CD8 depletion of E17 thymocytes by 80 ± 13% (from 33 ± 16% to 8 ± 8%, row five), in DN/CD8 transitional thymocytes prepared by CD4 depletion of E17 thymocytes by 36 ± 10% (from 66 ± 23% DP to 44 ± 20%, row six), in highly purified DN by 72% (from 39% DP to 11%) and 73% (from 40% DP to 10%) in two experiments (row seven), and in highly purified CD8 transitional thymocytes by 26% (from 91% DP to 67%) and 29%, (from 83% DP to 59%) in two experiments (row eight, all suspension culture experiments read out at 18 h). Recombinant BMPR-IA/Fc (bottom row, 1–3 μg/ml) increased the generation of DP cells in E15.5 thymic lobes cultured for 18 h by 67 ± 28% (from 23 to 38%, $n = 4$). (d) E15.5 thymus suspensions were cultured with 300 ng/ml of BMP4 or BMP7 and analyzed as in Fig. 3, b and c. BMP4 reduced the generation of DP cells by 73 ± 7% (from 50 ± 27% DP to 14 ± 9%) but BMP7 did not (1 ± 5%).

chanically prepared E15.5 thymocyte suspensions by 72 ± 8% (Fig. 3 c).

The ability of BMP4 to inhibit thymocyte differentiation in suspension cultures allowed us to define the BMP4 sensitivity of thymocytes at different developmental stages and, in addition, to ask whether BMP4 acts directly on thymocytes or via intermediate cell types. When E17 thymocyte suspensions were depleted of $CD8^+$ thymocytes to yield pure DN cells, BMP4 inhibited the generation of DP cells by 80 ± 13%. In contrast, when E17 thymocyte suspensions were depleted only of $CD4^+$ thymocytes, leaving behind DN as well as CD8 transitional cells, BMP4 was less effective at inhibiting the generation of DP cells (36 ± 10%). Hence, DN thymocytes were more sensitive to BMP4 than CD8 transitional cells (Fig. 3 c).

To determine whether BMP4 affects thymocyte development directly or indirectly, DN thymocytes were highly purified by two rounds of cell sorting for $HSA^{high}CD4^-CD8^-$ thymocytes, yielding populations that contained 0.3% or less of cell types other than thymocytes. Sorted thymocytes differentiated efficiently to the DP stage and BMP4 inhibited this by 72% and 73% in two independent experiments. The developmental progression of highly purified CD8 transitional cells was blocked by BMP4 to a lesser degree, by 26 and 29% in two independent experiments (Fig. 3 c). Hence BMP4 acts directly on DN thymocytes to inhibit their progression to the DP stage.

We exploited the observation that BMP2 and 4 (and to a lesser extent BMP7) but not TGFβ or activin can bind BMPR-IA with high affinity in the absence of type II receptor subunits (38, 39) to address whether the DN to DP transition is affected by endogenous BMPs. Soluble BMP receptor-IA subunit (BMPR-IA) was added to intact E15.5 thymic lobes in organ culture. In the presence of BMPR-IA, the percentage of DP thymocytes (analyzed 18 h later instead of 24–36 h in the organ culture experiments described above) was elevated by 67 ± 28% relative to control thymi (Fig. 3 c) and the number of DP cells per thymic lobe increased by 94 ± 57% (not shown).

Exogenous BMP2 blocked the DN to DP transition to the same extent as BMP4 (not shown). In contrast, BMP7 did not inhibit the DN to DP transition (Fig. 3 d), even at very high concentrations (1,000 ng/ml, not shown).

Smad-2 Phosphorylation in Response to TGFβ but Not BMP4 in Thymocytes. Like BMP4, TGFβ blocks thymocyte cell cycle activity and developmental progression (10, 40). To address the question whether thymocytes can distinguish BMP4 from TGFβ signals we analyzed the phosphorylation status of Smad-2, a downstream mediator of TGFβ signals (21) in thymocytes cultured with TGFβ1 at 1 ng/ml or a range of BMP4 concentrations from 0.1 to 1,000 ng/ml. Phosphorylated Smad-2 was readily detected in response to TGFβ but not to BMP4 by Western blotting with pSmad-2–specific antibodies (Fig. 4). Hence, BMP4 signaling does not appear to utilize the canonical TGFβ signaling pathway in thymocytes. An analysis of Smad-1 phosphorylation in response to exogenous BMP4 remained inconclusive as pSmad-1 appeared to be present in freshly isolated thymocytes (not shown, and see Discussion).

Tsg Synergizes with the BMP Inhibitor Chordin to Block BMP4 Effects on Thymocyte Differentiation. As described in Fig. 3 c, BMP4 inhibited the developmental progression from the DN to DP stage in Rag1$^{o/o}$ organ cultures treated with CD3ε. In the presence of BMP4 (100 ng/ml) the BMP inhibitor chordin restored the generation of DP cells only partially, even when added in threefold molar excess (2 μg/ml). We used this system to assess the functional effects of Tsg. On its own (not shown) or in combination with BMP4 (100 ng/ml), Tsg (1 μg/ml, ~40 nM) had little effect. In combination with chordin, however, TSG was able to reverse the inhibitory effects of BMP4 on the generation of DP thymocytes in anti-CD3ε–treated Rag1$^{o/o}$ organ cultures (Fig. 5 a, top panel). Rescue of DP differentiation from BMP4-mediated inhibition by chordin and Tsg was also seen in trypsinized suspension cultures of E15.5 thymi. Again, chordin antagonized BMP4 only partially, Tsg on its own was without effect, but the combination of chordin and Tsg rescued DP thymocyte development effectively (Fig. 5 a, bottom panel). A representative experiment is shown in Fig. 5 b.

The ratio between chordin and Tsg was found critical for BMP antagonism read out as secondary axis formation in zebrafish (19). We therefore performed experiments in which the concentrations of BMP4 (100 ng/ml) and chordin (2 μg/ml) were kept constant and Tsg was titrated over a wide range of concentrations, from 0.03 to 3,000 ng/ml (Fig. 5 b). Tsg addition resulted in a dose-dependent inhibition of the BMP4 block on the DN to DP transition. We conclude that Tsg can synergize with chordin to antagonize BMP4-mediated inhibition of the DN to DP transition. In this system there was no indication that Tsg had BMP-agonistic effects at any ratio of chordin and Tsg tested.

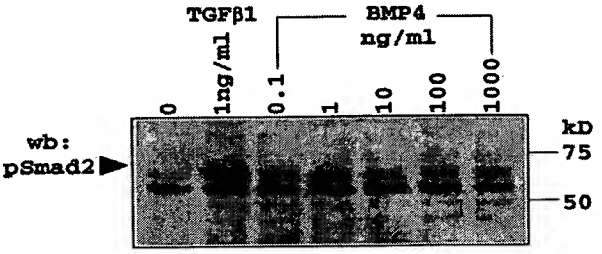

Figure 4. TGFβ but not BMP4 induce Smad-2 phosphorylation in thymocytes. Thymocytes from E17 (or postnatal, not shown) wild-type (C57BL/6) mice were cultured with TGFβ1 (1 ng/ml) or the indicated concentrations of BMP4 for 45 min and cell lysates assayed for pSmad-2 by Western blot.

Discussion

The key observation of our study is that immature thymocytes express Tsg, an extracellular modifier of BMP2/4 activity, in a developmentally regulated fashion: pre-TCR and TCR signaling results in increased Tsg expression at two developmental checkpoints, the DN to DP and the DP to SP transition (Fig. 1). Tsg has recently been shown to interact with extracellular components of the BMP signaling pathway (15–20), prompting us to investigate effects of BMP4 on thymocyte development. We found that DN thymocytes are highly susceptible to BMP4, which acts directly and without relay by another cell type to reduce DN thymocyte proliferation and progression to the DP stage in vitro (Fig. 3 c). The thymocyte response to BMP4 appears not to utilize the canonical TGFβ signaling cascade as indicated by the phosphorylation of Smad-2 in response to TGFβ but not to BMP4 (Fig. 4).

The addition of specific BMP inhibitors to intact thymic lobes results in elevated rates of thymocyte proliferation (Fig. 3 a) and accelerated pre-TCR dependent differentiation to the DP stage (Fig. 3 c), indicating that a BMP-imposed 'brake' may be active in situ. Conversely, exogenous BMP4 interferes with DN proliferation progression to the DP stage (Fig. 3, a–d), suggesting that endogenous BMP2/4 levels are subsaturating, or, alternatively, that not all thymocytes are exposed to equal BMP2/4 levels in situ. The latter possibility is consistent with our analysis of thymic sections where (in contrast to the uniform expression of BMP in early embryos; for a review, see reference 23) we found marked regional differences: BMP2/4 was seen predominantly in subcapsular and medullary areas and showed an uneven, 'patchy' distribution within these areas (Fig. 2, c and e). By analogy to the *Drosophila* ovary where the

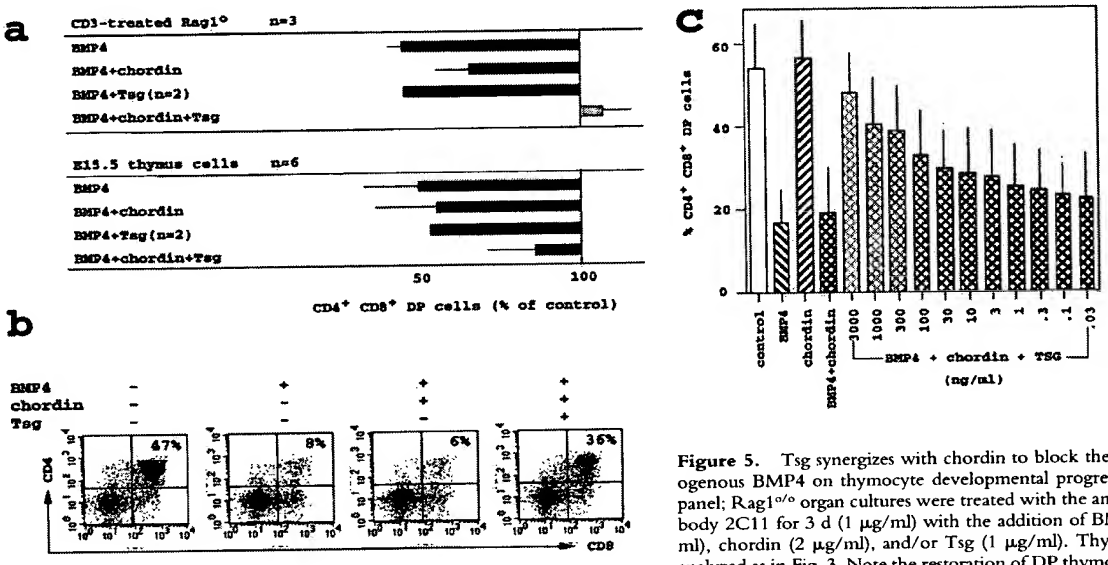

Figure 5. Tsg synergizes with chordin to block the effects of exogenous BMP4 on thymocyte developmental progression. (a) Top panel; Rag1$^{o/o}$ organ cultures were treated with the anti-CD3ε antibody 2C11 for 3 d (1 μg/ml) with the addition of BMP4 (100 ng/ml), chordin (2 μg/ml), and/or Tsg (1 μg/ml). Thymocytes were analyzed as in Fig. 3. Note the restoration of DP thymocyte development by the combination of chordin and Tsg. Bottom panel; suspension cultures of wild-type E15.5 thymi cultured overnight with the same additions as in top panel. (b) Suspension cultures of wild-type E15.5 thymi were cultured overnight and analyzed as in a. (c) E15.5 thymus suspensions were treated with BMP4 (100 ng/ml, left diagonal pattern), chordin (2 μg/ml, right diagonal pattern), or BMP4 plus chordin (cross-hatched) with or without Tsg (0.03 to 3,000 ng/ml) and analyzed as in Fig. 3. The percentage of DP thymocytes generated after 24 h is given (mean ± SD, $n = 4$).

BMP2/4 ortholog dpp serves to retain germ cell precursors in a concentration-dependent fashion (41) one might speculate that BMP2/4-rich areas represent specialized microenvironments geared toward the retention of precursor cells. In contrast to BMP2/4, the expression of BMP7 (which together with BMP5 and -6 forms the 60A subgroup of BMPs distinct from BMP2/4; reference 23) is not restricted to thymic stroma and BMP7 is expressed abundantly in thymocytes (Fig. 2 a). Interestingly, exogenous BMP7 did not inhibit the DN to DP transition (Fig. 3 d). Differences in the response to BMP2/4 and 7 have previously been seen in developing neurons (42, 43). Both BMP2/4 and BMP7 signaling involve Smad-1 (21, 23) and it is unclear how developing neurons (42, 43) or thymocytes (this study) discriminate between these BMPs. BMP7 may contribute to pSmad-1 seen in freshly isolated thymocytes (data not shown). In addition to BMP2/4 (this paper), TGFβ1 and the morphogen sonic hedgehog (Shh) can block the pre-TCR–dependent transition from the DN to the DP stage in vitro (9, 40). The relationship between BMP, Shh, and Wnt signals in the thymus remains to be defined. In certain developmental contexts Shh acts upstream of dpp/BMP2/4 (27, 41, 44–47). Conversely, Wnt and its downstream effectors (which can promote thymocyte differentiation; references 11 and 12) have been shown to block BMP4 expression in *Xenopus* embryos (48).

Reportedly, Tsg can either facilitate or antagonize dpp/BMP2/4 activity (15–20), perhaps because the release of dpp/BMP2/4 from the extracellular inhibitors sog/chordin is alternatively blocked (16) or facilitated (20) by the proteolytic processing of sog/chordin by metalloproteases of the tolloid/BMP1 family. The relative abundance of Tsg and chordin can be critical for whether Tsg acts as an agonist or an antagonist of dpp/BMP2/4 (17). In our own experiments Tsg synergized with chordin to antagonize BMP4 in a simple, dose-dependent manner (Fig. 5). If BMP2/4 originating from thymic stroma can block the expansion and differentiation of DN thymocytes in vivo, the developmentally regulated expression of Tsg could serve to temporarily antagonize inhibitory BMP effects following successful TCRβ rearrangement and pre-TCR expression

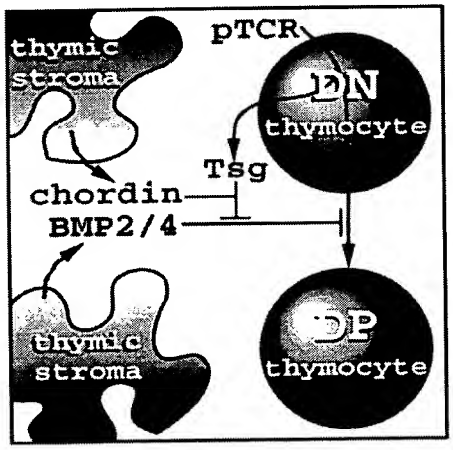

Figure 6. How BMP4, chordin, and Tsg may affect the pre-TCR–dependent DN to DP transition (see text for details).

(Fig. 6). We speculate that the balance between BMP2/4, chordin, and Tsg may ensure developmental progression while maintaining a sufficient pool of immature precursors. Although these ideas are consistent with our data on the developmental regulation of Tsg expression in vivo and our experiments in vitro, we emphasize that BMPs may have additional functions in the thymus. For example, a role at the DP stage is implied by differential Tsg expression in response to TCR engagement at the DP stage (Fig. 1) and the recently reported failure of thymocyte differentiate to the SP stage in the absence of Schnurri2, a putative downstream target of BMP signaling (49). Moreover, BMP4 and dpp act as morphogens in vertebrate and *Drosophila* embryos where they form activity gradients to specify distinct cell fates along the dorsal/ventral axis (23, 50–53). It will therefore be of interest to find out whether local BMP2/4 concentration specifies alternative fates such as αβ versus γδ at the DN to DP transition or CD4 versus CD8 at the DP to SP transition.

Our finding of developmentally regulated *Tsg* expression in the thymus extends the concept that cells within a morphogenetic field not only read and respond to the local morphogen concentration but can be instrumental in shaping the morphogen gradient (52, 53). It suggests that cells can temporarily withdraw from signaling molecules affecting their differentiation via the increased expression of a secreted modifier at specific developmental control points.

We thank Drs. Les Dale and Peter ten Dijke for discussions, constructs, and antibodies, Jonathan Carter for help with the mTsg-HA construct, Katy Smith for cell sorting, Spiros Lalos for help with histochemistry, and Dimitris Kontoyannis for comments on the manuscript.

Supported by the Medical Research Council, UK. D. Graf is currently the recipient of a Marie Curie Fellowship and would like to thank George Kollias for encouragement and support. D.B. Palmer is the recipient of a Medical Research Council Career Development Award.

Submitted: 20 February 2002
Revised: 29 April 2002
Accepted: 30 May 2002

References

1. Kisielow, P., and H. von Boehmer. 1995. Development and selection of T cells: facts and puzzles. *Adv. Immunol.* 58:87–209.
2. Levelt, C.N., and K. Eichmann. 1995. Receptors and signals in early thymic selection. *Immunity.* 3:667–672.
3. Jameson, S.C., K.A. Hogquist, and M.J. Bevan. 1995. Positive selection of thymocytes. *Annu. Rev. Immunol.* 13:93–126.
4. Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, and D.E. Williams, C.B. Ware, et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J. Exp. Med.* 180:1955–1960.
5. Rodewald, H.R., M. Ogawa, C. Haller, C. Waskow, and J.P. DiSanto. 1997. Pro-thymocyte expansion by c-kit and the common cytokine receptor gamma chain is essential for repertoire formation. *Immunity.* 6:265–272.
6. Zúñiga-Pflücker, J.C., D. Jiang, and M.J. Lenardo. 1995. Requirement for TNF- and IL-1 in fetal thymocyte commitment and differentiation. *Science.* 268:1906–1909.
7. Simpson, P. 1997. Notch signaling in development. *Perspect. Dev. Neurobiol.* 4:297–304.
8. MacDonald, H.R., A. Wilson, and F. Radtke. 2001. Notch1 and T-cell development: insights from conditional knockout mice. *Trends Immunol.* 22:155–160.
9. Outram, S.V., A. Varas, C.V. Pepicelli, and T. Crompton. 2000. Hedgehog signaling regulates differentiation from double-negative to double-positive thymocyte. *Immunity.* 13: 187–197.
10. Letterio, J.L., and A.B. Roberts. 1998. Regulation of immune responses by TGF-β. *Annu. Rev. Immunol.* 16:137–161.
11. Staal, F.J., J. Meeldijk, P. Moerer, P. Jay, B.C. van de Weerdt, S. Vainio, G.P. Nolan, and H. Clevers. 2001. Wnt signaling is required for thymocyte development and activates Tcf-1 mediated transcription. *Eur. J. Immunol.* 31:285–293.
12. Gounari, F., I. Aifantis, K. Khazaie, S. Hoeflinger, N. Harada, M.M. Taketo, and H. von Boehmer. 2001. Somatic activation of beta-catenin bypasses pre-TCR signaling and TCR selection in thymocyte development. *Nat. Immunol.* 2:863–869.
13. Graf, D., A.G. Fisher, and M. Merkenschlager. 1997. Rational primer design greatly improves differential display-PCR (DD-PCR). *Nucleic Acids Res.* 25:2239–2241.
14. Graf, D., P.M. Timmons, M. Hitchins, V. Episkopou, G. Moore, T. Ito, A. Fujiyama, A.G. Fisher, and M. Merkenschlager. 2001. Evolutionary conservation, developmental expression, and genomic mapping of mammalian Twisted gastrulation. *Mamm. Genome.* 12:554–560.
15. Oelgeschläger, M., J. Larrain, D. Geissert, and E.M. De Robertis. 2000. The evolutionary conserved BMP-binding protein Twisted gastrulation promotes BMP signalling. *Nature.* 405:757–763.
16. Yu, K., S. Srinivasan, O. Shimmi, B. Biehs, K.E. Rashka, D. Kimelman, M.B. O'Connor, and E. Bier. 2000. Processing of the Drosophila Sog protein creates a novel BMP inhibitory activity. *Development.* 127:2143–2154.
17. Scott, I.C., I.L. Blitz, WN. Pappano, S.A. Maas, KW. Cho, and D.S. Greenspan. 2001. Homologues of Twisted gastrulation are extracellular cofactors in antagonism of BMP signalling. *Nature.* 410:475–478.
18. Chang, C., D.A. Holtzman, S. Chau, T. Chickering, E.A. Woolf, LM. Holmgren, J. Bodorova, D.P. Gearing, W.E. Holmes, and A.H. Brivanlou. 2001. Twisted gastrulation can function as a BMP antagonist. *Nature.* 410:483–487.
19. Ross, J.J., O. Shimmi, P. Vilmos, A. Petryk, H. Kim, K. Gaudenz, S. Hermanson, S.C. Ekker, M.B. O'Connor, and J.L. Marsh. 2001. Twisted gastrulation is a conserved extracellular BMP antagonist. *Nature.* 410:479–483.
20. Larrain, J., M. Oelgeschlager, N.I. Ketpura, B. Reversade, L. Zakin, and E.M. De Robertis. 2001. Proteolytic cleavage of Chordin as a switch for the dual activities of Twisted gastrulation in BMP signaling. *Development.* 128:4439–4447.
21. Massague, J. 1998. TGFβ signal transduction. *Annu. Rev. Biochem.* 67:753–791.
22. Gorelik, L., and R.A. Flavell. 2000. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity.* 12:171–181.
23. Hogan, B.L.M. 1996. Bone morphogenetic proteins: multi-

functional regulators of vertebrate development. *Genes Dev.* 10:1580–1594.
24. Huber, T.L., and L.I. Zon. 1998. Transcriptional regulation of blood formation during Xenopus development. *Semin. Immunol.* 10:103–109.
25. Nakayama, N., J. Lee, and L. Chiu. 2000. Vascular endothelial growth factor synergistically enhances bone morphogenetic protein-4-dependent lymphohematopoietic cell generation from embryonic stem cells in vitro. *Blood.* 95: 2275–2283.
26. Marshall, C.J., C. Kinnon, and A.J. Thrasher. 2000. Polarized expression of bone morpho-genetic protein-4 in the human aorta-gonad-mesonephros region. *Blood.* 96:1591–1593.
27. Bhardwaj, G., B. Murdoch, D. Wu, D.P. Baker, K.P. Williams, K. Chadwick, L.E. Ling, F.N. Karanu, and M. Bhatia. 2001. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat. Immunol.* 2:172–180.
28. Scott, I.C., B.M. Steiglitz, T.G. Clark, W.N. Pappano, and D.S Greenspan. 2000. Spatiotemporal expression patterns of mammalian chordin during postgastrulation embryogenesis and in postnatal brain. *Dev. Dyn.* 217:449–456.
29. Dewulf, N., K. Verschueren, O. Lonnoy, A. Moren, S. Grimsby, K. Vande Spiegle, K. Miyazono, D. Huylebroeck, and P. Ten Dijke. 1995. Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. *Endocrinology.* 136: 2653–2663.
30. Dick, A., W. Risau, and H. Drexler. 1998. Expression of Smad1 and Smad2 during embryogenesis suggests a role in organ development. *Dev. Dyn.* 211:293–305.
31. Flanders, K.C., E.S. Kim, and A.B. Roberts. 2001. Immunohistochemical expression of Smads 1-6 in the 15-day gestation mouse embryo: signaling by BMPs and TGF-betas. *Dev. Dyn.* 220:141–154.
32. Mason, E.D., K.D. Konrad, C.D. Webb, and J.L. Marsh. 1994. Dorsal midline fate in drosophila embryos requires twisted gastrulation, a gene encoding a secreted protein related to human connective tissue growth factor. *Genes Dev.* 8:1489–1501.
33. Spanopoulou, E., P. Cortes, C. Shih, C.M. Huang, D.P. Silver, P. Svec, and D. Baltimore. 1995. Localization, interaction, and RNA binding properties of the V(D)J recombination-activating proteins RAG1 and RAG2. *Immunity.* 3: 715–726.
34. Koller, B.H., P. Marrack, J.W. Kappler, and O. Smithies. 1990. Normal development of mice deficient in beta 2m, MHC class I proteins, and CD8+ T cells. *Science.* 248:1227–1230.
35. Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis. 1991. Mice lacking MHC class II molecules. *Cell.* 66:1051–1066.
36. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97–104.
37. Merkenschlager, M., D. Graf, M. Lovatt, U. Bommhardt, R. Zamoyska, and A.G. Fisher. 1997. How many thymocytes audition for selection? *J. Exp. Med.* 186:1149–1158.
38. Natsume, T., S. Tomita, S. Iemura, N. Kinto, A. Yamaguchi and N. Ueno. 1997. Interaction between soluble type I receptor for bone morphogenetic protein and bone morphogenetic protein-4. *J. Biol. Chem.* 272:11535–11540.
39. Kawabata, M., T. Imamura, and K. Miyazono. 1998. Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev.* 9:49–61.
40. Takahama, Y., J.J. Letterio, H. Suzuki, A.G. Farr, and A. Singer. 1994. Early progression of thymocytes along the CD4/CD8 developmental pathway is regulated by a subset of thymic epithelial cells expressing transforming growth factor beta. *J. Exp. Med.* 179:1495–1506.
41. Xie, T., and A.C. Spradling. 1998. decapentaplegic is essential for the maintenance and division of germline stem cells in the Drosophila ovary. *Cell.* 94:251–260.
42. Furuta, Y., D.W. Piston, and B.L.M. Hogan. 1997. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development.* 124:2203–2212.
43. Shou, J., R.C. Murray, P.C. Rim, and A.L. Calof. 2000. Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage. *Development.* 127:5403–5413.
44. Xie, T., and A.C. Spradling. 2000. A niche maintaining germ line stem cells in the Drosophila ovary. *Science.* 290:328–330.
45. Laufer, E., C.E. Nelson, R.L. Johnson, B.A. Morgan, and C. Tabin. 1994. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell.* 79:993–1003.
46. Murtaugh, L.C., J.H. Chyung, and A.B. Lassar. 1999. Sonic hedgehog promotes somitic chondrogenesis by altering the cellular response to BMP signaling. *Genes Dev.* 13:225–237.
47. Zuniga, A., A.P. Haramis, A.P. McMahon, and R. Zeller. 1999. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature.* 401: 598–602.
48. Baker, J.C., R.S. Beddington, and R.M. Harland. 1999. Wnt signaling in Xenopus embryos inhibits bmp4 expression and activates neural development. *Genes Dev.* 13:3149–3159.
49. Takagi, T., J. Harada, and S. Ishii. 2001. Murine Schnurri-2 is required for positive selection of thymocytes. *Nat. Immunol.* 2:1048–1053.
50. Gurdon, J.B., S. Dyson, and D. St. Johnston. 1998. Cells' perception of position in a concentration gradient. *Cell.* 95: 159–162.
51. Dale, L. 2000. Pattern formation: a new twist to BMP signalling. *Curr. Biol.* 10:R671–R673.
52. Tabata, T. 2001. Genetics of morphogen gradients. *Nat. Rev. Genet.* 2:620–630.
53. Teleman, A.A., M. Strigini, and S.M. Cohen. 2001. Shaping morphogen gradients. *Cell.* 105:559–562.